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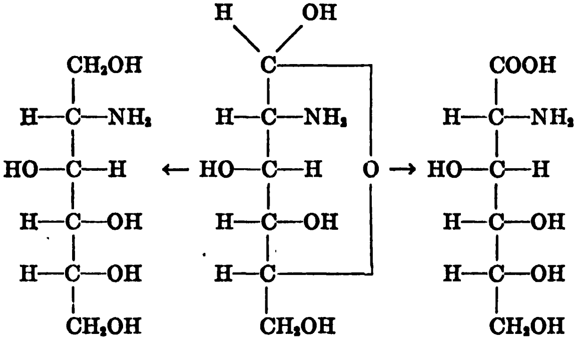
ON A CATALYTICALLY INDUCED REACTION RESEMBLING THE CANNIZZARO REACTION

By P. A. LEVENE and CLARENCE C. CHRISTMAN

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, June 25, 1937)

When glucosamine is agitated in an atmosphere of hydrogen in the presence of a platinum catalyst, only 50 per cent of the sugar is reduced to aminosorbitol and the remaining 50 per cent is oxidized to glucosaminic acid. The reaction proceeds in the following way.



This reaction resembles the well known Cannizzaro reaction which consists of an oxidation-reduction process leading from an aldehyde to equimolecular proportions of acid and carbinol. The Cannizzaro reaction is generally regarded as a homogeneous reaction catalyzed by hydroxyl ions. It is common in the field of aromatic chemistry, where it serves for preparative purposes, but it is not so common in the field of aliphatic aldehydes. In animal tissues and in plants there are present enzymes which have equal power to effect a Cannizzaro reaction in the aliphatic as well as in the aromatic aldehydes. The reaction plays a prominent part in the process of fermentation.

The resemblance of the reaction induced by a platinum catalyst to that induced by the biocatalysts called for a more detailed study of its mechanism.

Subsequently it was found that the products of the reaction produced by contact with platinum catalysts varied with changes in the external conditions, the hydrion concentration and the hydrogen gas pressure being the main factors influencing the course of the reaction. It should be mentioned that the mode of preparation of the catalyst has apparently no effect on the outcome of the reaction. Thus, Adams' catalyst—whether reduced or not prior to use—as well as platinum black prepared according to the directions of Willstätter, acted in a similar way. It also should be mentioned that on contact with the catalyst in the absence of hydrogen, glucosamine remained unchanged. Likewise, a reaction did not take place in an atmosphere of hydrogen in the absence of the catalyst. The latter fact shows that the hydrion concentration due to glucosamine of itself is not capable of catalyzing a Cannizzaro reaction.

Methods of Analysis—The quantity of acid formed was determined by titration to pH 4.5 and, inasmuch as volatile acids could not be detected in the reaction products, the increase of acidity was attributed to the formation of glucosaminic acid. The amount of isolated glucosaminic acid agreed as well as could be expected with that calculated by titration.

The loss in glucosamine was determined by titration according to Hanes-Hagedorn and Jensen.[1]

In all cases an aqueous solution of the substance and the catalyst were placed in the Adams' apparatus in an atmosphere of hydrogen. In all experiments save a few the pressure of the gas was 35 pounds per sq. inch; in the exceptional cases the pressure was either atmospheric or less.

The results of the experiments are given in Table I. From this table it can be seen that: (1) Free glucosamine yields approximately equimolecular proportions of glucosaminic acid and aminosorbitol. There seems to be a slightly higher production of aminosorbitol. When the hydrogen pressure was reduced to

[1] Hanes, C., *Biochem. J.*, 23, 99 (1929).

atmospheric, an excess of acid as compared with aminosorbitol was formed. (2) When glucosamine hydrochloride, or N-acetylglucosamine is subjected to the same treatment as in (1), then only one product is formed, namely aminosorbitol, or its acetate, the reduction of the N-acetylglucosamine proceeding at a considerably slower rate than that of the hydrochloride. (3) When the gas pressure in the apparatus is reduced below atmospheric pressure, the catalyst having been reduced prior to the introduction of the glucosamine solution—the solution containing, in addition to glucosamine, sodium hydroxide at 0.05 N concentration—then the product of the reaction is only glucosaminic acid.

TABLE I

Proportions of Acids and Polyhydric Amino Alcohols Formed from Glucosamine under Different External Conditions on Contact with Platinum Catalyst in an Atmosphere of Hydrogen

	Pressure per sq. in.	Glucosamine	Amino alcohol	Amino acid
		per cent	*per cent*	*per cent*
Glucosamine hydrochloride + Adams' catalyst + hydrogen gas	35 pounds	0	100	0
N-Acetylglucosamine + Adams' catalyst + hydrogen gas	35 "	0	100	0
Glucosamine + Adams' catalyst + hydrogen gas	35 "	0	53	47
Glucosamine + Adams' catalyst reduced + hydrogen gas	35 "	0	53	47
Glucosamine + Adams' catalyst + hydrogen gas + NaOH, 0.05 N	35 "	0	48	52
Glucosamine + Adams' catalyst reduced + hydrogen gas	Much reduced	16	3	81
Glucosamine + Adams' catalyst reduced + hydrogen gas + NaOH, 0.05 N	" "	0	0	100
Glucosamine + Adams' catalyst + hydrogen gas, *t* 8°, time 72 hrs	Atmospheric	0	46	54
Glucosamine + Adams' catalyst + hydrogen gas, *t* 67°, time 1 hr	"	0	44	56

Kinetics of Oxidation-Reduction Reaction—The progress of the reaction of free glucosamine in an atmosphere of hydrogen in contact with Adams' catalyst under 35 pounds pressure is given in

Table II. The progress of the reaction was measured by titrating the amount of acid formed (x), the amount of glucosamine remaining at time t being ($a - 2x$).

TABLE II

Rates of Formation of Glucosaminic Acid from Glucosamine on Contact with Platinum Catalyst in an Atmosphere of Hydrogen

Time	Volume of 0.020 N HCl to neutralize base present	Volume of 0.020 N acid formed during reaction x	$k = \frac{1}{t} \log \frac{a}{a - 2x}$
hrs.	*cc.*	*cc.*	
0	6.65 (a)	0.00	
1	5.21	0.72	0.1059
2	3.99	1.33	0.1109
3	4.05	1.80	0.1129
4	2.35	2.15	0.1129
5	1.85	2.40	0.1111
6	1.57	2.54	0.1046
7	1.41	2.62	0.0962
12	0.81	2.92	0.0762
24	0.41	3.02	0.0432
48	0.35	3.10	0.0244

TABLE III

Rate of Disappearance of Glucosamine

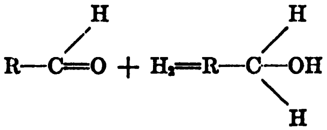
Time	0.01 N $Na_2S_2O_3 \propto$ sugar present	Sugar present, $a - x$	a	$k = \frac{1}{t} \log \frac{a}{a - x}$
hrs.	*ml.*	*mg.*		
0	9.09	3.05		
1	6.56	2.23	2.23	
2	5.32	1.81		0.0930
3	4.25	1.45		0.0934
4	2.75	0.95		0.0924
6	2.04	0.70		0.0838
10	1.36	0.47		0.0676

From Table II it may be seen that the reaction followed a monomolecular course until the reaction was over 80 per cent completed. In these experiments no special precautions were used to maintain constant temperature or constant pH. In a second experiment, the reaction was carried out at constant pH and constant temperature. However, the hydrogen pressure was reduced to 1 atmosphere. In this case the reaction seemed to follow a monomolecular course until about 70 per cent of the sugar was changed into glucosaminic acid and into aminosorbitol. The constancy of k_1 (Table III), however, is spurious for there was observed in every interval a noticeable higher production of acid over that of alcohol.

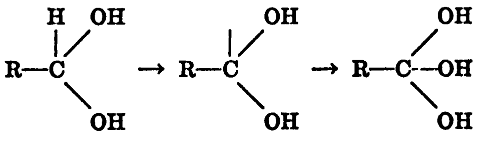
Effect of Temperature—Most of the experiments were performed at room temperature at a pressure of 35 pounds per sq. inch. For the study of the temperature effect an apparatus was constructed consisting of the conventional glass vessel (Ente) used for hydrogenation experiments, provided with a jacket for circulating water. The rate of reaction was measured at 8° and at 67°. In each case 56 per cent of glucosaminic acid and 44 per cent of the aminosorbitol were formed at the end of the experiment. This yield was obtained after 72 hours at the lower temperature and after 1 hour at the higher temperature. Hence the reaction approximately doubled for every increment of the temperature of 10°.

Discussion of Results

Inasmuch as the oxidation-reduction reaction of glucosamine does not take place in the absence of the catalyst, the reaction is one of heterogeneous catalysis. At the point of neutrality glucosamine functions as the acceptor of hydrogen and the catalyst as the donor. Thus it is possible that in the reaction of reduction the carbonyl group functions as any unsaturated group.

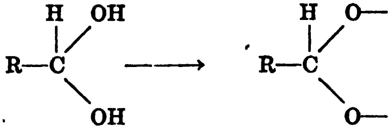


On the other hand, the reaction of oxidation requires the presence of hydroxyl groups. It is therefore possible that this reaction is preceded by the formation of the hydrated form of glucosamine (I)



I II III

which is dehydrogenated by the catalyst into (II), which, in its turn, accepts a hydroxyl group to form the acid. Thus, in the reaction of oxidation the catalyst acts as an acceptor of hydrogen. Whether this is the true mechanism or whether 2 hydrogen atoms are removed synchronously from the hydrated form (I),



III IV

as postulated by Wieland,[2] remains uncertain.

The combined oxidation-reduction reaction takes place when conditions are given for both reactions; namely, sufficient pressure of hydrogen gas to maintain a sufficient supply of platinum dihydride and a sufficient hydroxyl ion concentration to give rise to the hydrogenated form. It would then follow that each reaction takes place on a single molecule of the aldehyde and not on a bimolecular condensation product. Thus the reactions of reduction and oxidation are two separate reactions. The latter is dependent upon the first only to the extent as decrease in the mass of the platinum dihydride enhances the process of oxidation.

It may be of interest now to consider the extent to which the platinum catalyst may serve as a model of the enzymatic Cannizzaro reaction and perhaps even of the homogeneous Cannizzaro reaction.

Before we enter into this analysis, it should be pointed out that reactions similar to that described above have been observed with substances other than glucosamine, although, to our knowledge, the simultaneous oxidation and reduction have never been observed under the conditions described above. Simple monoses were reduced catalytically to corresponding polyhydric alcohols in this laboratory[3] and later by Glattfeld.[4] Oxidation of glucose to carbon dioxide was accomplished by Wieland with large excess of catalyst (2.7 gm. for 0.125 gm. of sugar) under a pressure of hydrogen much below atmospheric pressure.

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[2] Wieland, H., *Ber. chem. Ges.*, 46, 3327 (1913).

[3] Levene, P. A., and Harris, S. A., *J. Biol. Chem.*, 98, 9 (1932).

[4] Glattfeld, J. W. E., and Schimpff, G. W., *J. Am. Chem. Soc.*, 57, 2204 (1935).

A reaction resembling the Cannizzaro, that is equimolecular production of polyhydric alcohol and corresponding carboxylic acid from simple monoses, has been recently reported by Bach *et al.*[5] in 0.5 N solution of alkali and under a pressure of hydrogen much below atmospheric. Under such conditions glucosamine yields only glucosaminic acid. In the experiments by Bach *et al.* the *concentration of the alkali was about 10 times as high as in the experiments with glucosamine.* It is possible that the differences in the outcome of the reaction might be attributed to this cause.[6]

It may be mentioned here that simultaneous hydrogenation and dehydrogenation of unsaturated cyclic hydrocarbons was observed by Zeiling.[7] The reaction, however, took place at a higher temperature and in an atmosphere of carbon dioxide, thus again under conditions totally different from those of our experiment.

Comparison of Reactions Induced by Chemical Catalyst and by Biocatalyst—As far as it is known, the enzyme, though not isolated in pure form, is definitely a substance of high molecular weight with colloidal properties. Hence the reaction by contact with the enzyme may be regarded as a heterogeneous reaction. Similarly to the platinum catalyst, the biocatalyst is capable of producing either oxidation or reduction, as well as oxidation and reduction simultaneously. The hydrion concentration is an important factor influencing the rate and the outcome of the enzymatic reaction as it does in the reaction on the platinum catalyst.[8] Thus there is a possibility that a more detailed study of the enzymatic reaction may reveal greater similarity in the action of the two catalysts.

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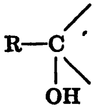
[5] Bach, A., Alekseeva, E. P., and Dreving, V. P., *Biochimia*, **1**, 75 (1936).

[6] The publication of Bach *et al.* in a new Russian journal reached us very recently, long after our observations were made. Our own observations were made in course of renewed efforts to determine the configuration of glucosamine. It was intended to reduce aminosorbitol to 2-aminohexane whose configuration has been correlated by Levene and Mardasheff to α-aminocaproic acid. Great difficulties, however, are being encountered in this work which accounts for the delay in publishing the present article.

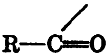
[7] Zeiling, A., *Ber. chem. Ges.*, **57**, 2055, 2058 (1924).

[8] Batelli, F., and Stern, L., *Biochem. Z.*, **28**, 159 (1910). Parnas, J., *Biochem. Z.*, **28**, 274 (1910). Quastel, S. H., and Wheatley, A. H. M., *Biochem. J.*, **26**, 2167 (1932). Batelli, F., and Stern, L., *Biochem. Z.*, **29**, 130 (1910).

Homogeneous and Heterogeneous Cannizzaro Reactions—Although the majority of workers accept complex formation as the first step of the homogeneous reaction of Cannizzaro,[9] yet there are authors accepting dehydrogenation as the first step of the reaction with the formation either of a divalent carbon compound[10]



or of a free radicle[11]



Furthermore, it has been observed by Varley[12] that the reaction of Cannizzaro may be shifted by external conditions towards production of the carbinol in excess of the acid. Recently Kuhn and Meyer[13] produced evidence to show that in a medium free from contamination ("sterile") autoxidation of benzaldehyde does not take place. Thus there is not excluded the possibility that a catalyst other than the hydroxyl ion induces the so called homogeneous Cannizzaro reaction.

EXPERIMENTAL

Reduction of Free Glucosamine—10 gm. of free glucosamine were dissolved in 200 cc. of water (distilled from glass vessels) and to this solution 1 gm. of Adams' catalyst was added. The mixture was shaken with hydrogen, at a pressure of 35 pounds per sq. inch, for 2 days, at the end of which time the solution failed to reduce boiling Fehling's solution.

The catalyst was removed by filtration, washed well with water, and then kept in the refrigerator for further reductions. The filtrate and washings were combined and concentrated under reduced pressure at 40° to about 25 to 30 cc.

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[9] Tischtschenko, W., *J. Russ. Physic.-Chem. Soc.*, **38**, 482 (1937). Claisen, L., *Ber. chem. Ges.*, **20**, 646 (1887). Kohn, C. A., and Trantom, W. J., *J. Chem. Soc.*, **75**, 1155 (1899). Lachman, A., *J. Am. Chem. Soc.*, **45**, 2358 (1923). Merwein, H., and Schmidt, R., *Ann. Chem.*, **444**, 221 (1925).

[10] Nef, J. V., *Ann. Chem.*, **298**, 301 (1897).

[11] Haber, F., and Willstätter, R., *Ber. chem. Ges.*, **64**, 2844 (1931).

[12] Varley, A., *Bull. Soc. chim.*, **87**, 537 (1925).

[13] Kuhn, R., and Meyer, K., *Naturwissenschaften*, **16**, 1028 (1928).

The experiment from this point is divided into two parts; namely, *A. Isolation of glucosaminic acid* and *B. Preparation of crystalline 2-aminosorbitol hexaacetate.*

A. Isolation of Glucosaminic Acid

Absolute ethyl alcohol was then added to the above concentrated solution until crystallization was complete. After the material stood in the refrigerator for several hours, the crystals were removed by filtration and washed first with cold 85 per cent alcohol and then with absolute alcohol. The mother liquor and washings were concentrated under reduced pressure to about 20 cc. and from this concentrate another crop of crystals was obtained. In all, four crops of crystals were obtained in this way. Yield, 4.0 gm. (The filtrates and washings were concentrated to a thick sirup and kept in the refrigerator. The isolation of a product from this material will be discussed in Part B.)

The product was recrystallized five times by dissolving in a small amount of water and then adding absolute ethyl alcohol. This gave a pure material having the following specific rotation

$$[\alpha]_D^{25} = \frac{-1.10° \times 100}{1 \times 8.24} = -13.4° \text{ (in 20\% hydrochloric acid)}$$

The substance had the following composition.

5.412 mg. substance: 7.305 mg. CO_2 and 3.170 mg. H_2O
5.400 " " : 0.352 cc. N_2 (760 mm. at 25°)
$C_6H_{13}O_6N$. Calculated. C 36.91, H 6.8, N 7.6
Found. " 36.75, " 6.5, " 7.5

B. Preparation of Crystalline 2-Aminosorbitol Hexaacetate

The sirupy residue remaining after the removal of the glucosaminic acid was dried to constant weight by concentrating under reduced pressure with small portions of benzene. The sirup was acetylated by warming and triturating for 1 hour with a mixture of 30 cc. of acetic anhydride and 30 cc. of dry pyridine. At the end of this time the sirup had gone into solution, the solution having a brown color. The mixture was kept at room temperature overnight and then poured into ice and water.

The water solution was now extracted with three portions of chloroform and the chloroform extract washed successively with water, 5 per cent sulfuric acid, sodium bicarbonate solution, and finally with water. It was dried with anhydrous sodium sulfate and concentrated under reduced pressure, at 40°, to a thick sirup. All attempts to crystallize this sirup failed.

The dried sirup was therefore distilled under a high vacuum and a yellow viscous sirup was collected distilling between 160–180° (bath temperature 200–210°) and 0.3 mm. Yield, 8.0 gm. This sirup started to crystallize after standing at room temperature in an open test-tube for several weeks. When the main portion of the sirup was dissolved in a small volume of absolute ethyl alcohol, and an equal volume of ether and of pentane added until the solution became opalescent, followed by nucleation with the crystals, the substance settled out in crystalline form. After two more recrystallizations, the substance was analytically pure. Yield, 4.5 gm. The substance melted at 99–100° and had the following specific rotation.

$$[\alpha]_D^{25} = \frac{+1.83^\circ \times 100}{2 \times 4.36} = +21.0^\circ \text{ (in chloroform)}$$

The substance is soluble in ethyl alcohol, methyl alcohol, acetone, water, benzene, chloroform, and ethyl acetate. It is moderately soluble in ether and is practically insoluble in pentane.

The substance had the following composition.

4.652 mg. substance: 8.510 mg. CO_2 and 2.580 mg. H_2O
9.198 " " : 0.263 cc. N_2 (771 mm. at 25°)
7.271 " " : 9.843 " 0.01 N $Na_2S_2O_3$[14]
$C_{18}H_{27}O_{11}N$. Calculated. C 49.90, H 6.3, N 3.2, $COCH_3$ 58.2
Found. " 49.89, " 6.2, " 3.3, " 58.2

Preparation of N-Acetyl-2-Aminosorbitol—15 gm. of pure 2-aminosorbitol hexaacetate were dissolved in 250 cc. of absolute methyl alcohol and the solution cooled in an ice-alcohol mixture. 10 cc. of 0.5 N barium methylate solution in absolute methyl alcohol[15] were then added and the mixture kept in the refrigerator for

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[14] Elek, A., and Harte, R. A., *Ind. and Eng. Chem., Anal. Ed.*, 8, 267 (1936).

[15] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, 93, 631 (1931).

2 days during which time a crystalline deposit formed. This was filtered off and washed with cold absolute methyl alcohol. Yield, 2.0 gm.

The filtrate and washings were diluted with 120 cc. of water and the solution was saturated with carbon dioxide. The solution was decolorized by boiling with charcoal. The filtrate was concentrated under reduced pressure at 40° and the residue recrystallized from absolute ethyl alcohol giving another crop of crystals weighing 4.5 gm. Total yield, 6.5 gm.

The substance was pure after three recrystallizations from absolute ethyl alcohol and had a melting point of 152–153°. Its specific rotation was

$$[\alpha]_D^{25} = \frac{-0.93^\circ \times 100}{2 \times 4.492} = -10.4^\circ \text{ (in water)}$$

The substance is soluble in warm ethyl or methyl alcohol and water. It is practically insoluble in ether, acetone, chloroform, and 30–40° petroleum ether.

The substance had the following composition.

4.912 mg. substance: 7.780 mg. CO_2 and 3.195 mg. H_2O
25.30 " " : 0.00 cc. amino N_2 (757 mm. at 27°)
$C_{18}H_{17}O_6N$. Calculated. C 43.10, H 7.7, amino N 0.00
Found. " 43.19, " 7.82, " " 0.00

Action of Adams' Catalyst on Free Glucosamine in Absence of Hydrogen[16]—1 gm. of free glucosamine was dissolved in 150 cc. of water (distilled from glass vessels) and to this 0.2 gm. of unreduced Adams' catalyst was added. This was shaken in the Adams' shaking machine, in air atmosphere, for 2 days. A 5 cc. portion of the solution at the beginning of the reaction had the same reducing power as a 5 cc. portion at the end of the 2nd day, thus indicating that no reaction had taken place.

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[16] In this and the following experiments the formation of amino acid was determined by titration with 0.02 N hydrochloric acid to pH 4.5, with sodium alizarin sulfonate as an indicator. In the experiments to which alkali was added, the number of ml. of 0.02 N acid required to neutralize this alkali was deducted from the total volume used. At the end of each experiment the concentration of remaining unchanged glucosamine was determined by the Hanes-Hagedorn and Jensen method.[1]

Action of Hydrogen Gas on Free Glucosamine in Absence of Adams' Catalyst—1 gm. of free glucosamine was dissolved in 150 cc. of water (distilled from glass vessels) and then shaken in the Adams' shaking machine with hydrogen at a pressure of 35 pounds per sq. inch for 2 days. The reducing power of equal portions taken at the beginning and at the end of the experiment was identical.

Action of Adams' Catalyst in an Atmosphere of Hydrogen on Glucosamine Hydrochloride—1 gm. of pure glucosamine hydrochloride was dissolved in 100 cc. of water (distilled from glass vessels) and 100 mg. of Adams' catalyst were added. The acid content and reducing sugar content in aliquot portions of the solution were then estimated.

The mixture was now shaken with hydrogen at a pressure of 35 pounds per sq. inch for 3 days, at the end of which time a test portion of the solution failed to reduce boiling Fehling's solution. The titration of aliquot portions of this final solution revealed that no acid was formed during the reaction and that no reducing sugars were present.

The catalyst was removed by filtration and the filtrate concentrated to dryness under diminished pressure at 40°. The sirup was crystallized by dissolving in 2 cc. of water and adding 15 cc. of absolute ethanol. Two crops of crystals were obtained in this way. Yield, 0.65 gm. (theoretical yield, 0.70 gm., since 30 cc. of the above reaction mixture had been used for titrations).

The substance was pure after three recrystallizations from water-alcohol and had a melting point of 157–158° and the following specific rotation.

$$[\alpha]_D^{25} = \frac{-0.21^\circ \times 100}{2 \times 4.4} = -2.4^\circ \text{ (in 20\% hydrochloric acid)}$$

The substance is soluble in water and practically insoluble in the usual organic solvents.

It had the following composition.

5.203 mg. substance: 6.310 mg. CO_2 and 3.310 mg. H_2O
5.500 " " : 0.309 cc. N_2 (760 mm. at 27°)
$C_6H_{16}O_5NCl$. Calculated. C 33.10, H 7.4, N 6.4
Found. " 33.07, " 7.1, " 6.4

Action of Adams' Catalyst in an Atmosphere of Hydrogen on N-Acetylglucosamine—N-Acetylglucosamine (0.80 gm.) was dissolved in 100 cc. of water (distilled from glass vessels) and 200 mg. of Adams' catalyst were added. The acid content and reducing sugar in aliquot portions of the solution were then determined.

The mixture was now shaken with hydrogen at a pressure of 35 pounds per sq. inch for 2 days. The titration of aliquot portions of this solution indicated the total absence of acid and the presence of about 20 per cent reduced material, thus indicating the absence of any oxidation.

Another 100 mg. of catalyst were then added to the above mixture every 24 hours until, at the end of the 10th day, the reduction was complete, since a portion of the final solution failed to reduce boiling Fehling's solution. Titration of an aliquot portion showed the absence of any acid.

TABLE IV

Experimental Data Showing Acid Formation Titrations

2.5 ml. were taken for each titration.

Experiment No.	0.02 N HCl required for titration of sample		Acid formed = $\frac{\text{ml. initial} - \text{ml. final}}{\text{ml. initial}} \times 100$	Unchanged glucosamine
	Initial	Final		
	ml.	*ml.*	*per cent*	*per cent*
I	6.60	3.50	47	0
II	12.85 − 6.20* = 6.65	9.40 − 6.20* = 3.20	52	0
III	6.63	1.28	81	16
IV	12.65 − 6.20* = 6.45	6.25 − 6.20* = 0.05	100	0
V	6.68	3.05	54	0
VI	6.67	2.95	56	0

* Number of ml. of alkali added.

Action of Reduced Adams' Catalyst in an Atmosphere of Hydrogen on Free Glucosamine—Adams' catalyst (100 mg.) was added to 50 cc. of water (distilled from glass vessels) and then shaken with hydrogen in order to reduce the catalyst completely. 500 mg. of free glucosamine were introduced into the apparatus.

This mixture was now shaken with hydrogen at a pressure of 35 pounds per sq. inch for 2 days. At the end of that time test portions of the solution failed to reduce boiling Fehling's solution. Titration of aliquot portions indicated the formation of 47 per cent acid (Table IV, Experiment I). The remainder was amino alcohol, since a portion of the final solution did not reduce boiling Fehling's solution. These results are the same as in previous experiments in which the catalyst was not reduced prior to the addition of the glucosamine.

Action of Platinum Black in an Atmosphere of Hydrogen on Free Glucosamine—1 gm. of free glucosamine was dissolved in 100 cc. of water (distilled from glass vessels) and 100 mg. of platinum black were added. The mixture was then shaken with hydrogen at a pressure of 35 pounds per sq. inch for 2 days at the end of which time a test portion of the solution failed to reduce boiling Fehling's solution.

The catalyst was removed by filtration and the filtrate concentrated to a sirup under reduced pressure at 40°. The sirup was dissolved in a small volume of water and absolute ethanol was added until no further increase in the crystalline deposit was observed. It was then placed in the refrigerator until crystallization was complete. (Total yield, 0.40 gm.) These crystals had a composition corresponding to that for glucosaminic acid. (Calculated, C 36.91, H 6.8; found, C 37.13, H 7.05.) The amino alcohol in the alcoholic filtrates was not isolated, since it had been examined in previous experiments.

Action of Adams' Catalyst in an Atmosphere of Hydrogen on Free Glucosamine in Presence of Alkali—Free glucosamine (0.500 gm.) was added to a solution of 25 ml. of water and 25 ml. of 0.1 N NaOH. Then Adams' catalyst (100 mg.) was introduced into the apparatus and the mixture was shaken with hydrogen at a pressure of 35 pounds per sq. inch for 24 hours.

Titrations taken at the beginning and after the elapse of 24 hours indicated the formation of 52 per cent acid (Table IV, Experiment II). The remainder was amino alcohol (no reduction of Fehling's solution by the final solution).

Action of Reduced Adams' Catalyst on Free Glucosamine under Reduced Pressure—To 100 ml. of water (distilled from glass vessels) 100 mg. of Adams' catalyst were added and the mixture shaken with hydrogen for 20 minutes in order to reduce the catalyst completely. Free glucosamine (1 gm.) was then introduced and the air of the apparatus was replaced by hydrogen gas which was then evacuated.

The mixture was next shaken for 24 hours. Titrations of aliquot portions of this solution at the beginning and after the elapse of 42 hours indicated the formation of 81 per cent acid (Table IV, Experiment III) and only a small amount of amino alcohol, since only 84 per cent of the sugar present was reduced. (A 1 ml. portion showed the presence of 3.03 mg. of sugar at the beginning of the reaction and 0.46 mg. at the end of the reaction.)

Action of Adams' Catalyst on Free Glucosamine and Alkali under Reduced Pressure—To 25 cc. of water, Adams' catalyst (100 mg.) was added and this mixture shaken with hydrogen for 20 minutes. The bottle was then opened and 500 mg. of free glucosamine were added together with 25 ml. of 0.1 N sodium hydroxide solution. Hydrogen was now quickly passed into the apparatus and then the whole apparatus evacuated for 10 minutes. This mixture was next shaken under reduced pressure for 72 hours at the end of which time the solution failed to reduce boiling Fehling's solution.

Titrations of aliquot portions of the solution taken at the beginning and after the elapse of 72 hours indicated the complete transformation of the glucosamine into glucosaminic acid (Table IV, Experiment IV) without any noticeable formation of amino alcohol.

Rate of Formation of Glucosaminic Acid in the Reaction between Free Glucosamine and Adams' Catalyst and Hydrogen at 25°—1 gm. of free glucosamine was dissolved in 100 cc. of water (distilled from glass vessels) and 100 mg. of Adams' catalyst were added. This mixture was then shaken with hydrogen at a pressure of 35 pounds per sq. inch at 25°.

A portion (2.5 cc.) was removed from the reaction mixture at regular intervals and titrated with 0.020 N hydrochloric acid, with sodium alizarin sulfonate as indicator. The data obtained and the value of k, calculated for a first order reaction, are given in Table II. The amount of acid formed was approximately 47 per cent.

Comparison of Rates of Acid Formation at 8° and 67° in Reaction of Free Glucosamine, Adams' Catalyst, and Hydrogen—Free glucosamine (0.5000 gm.) was dissolved in 50 cc. of water (distilled from glass vessels) and 100 mg. of Adams' catalyst were added. The apparatus was enclosed in a water jacket through which water, at a temperature of 67° in one case and 8° in the other, circulated.

The mixtures were shaken with hydrogen at *atmospheric pressure* until all reducing sugar disappeared. Titrations on aliquot portions of the solutions were taken and the amount of acid formed in a given time tabulated. The amount of acid formed at 8° and at 67° was approximately 56 per cent (Table IV, Experiment V and Experiment VI respectively) and this amount of acid was formed in approximately 1 hour at 67° and in approximately 72 hours at 8°.

Rate of Reaction of Glucosamine in Buffered Solution in Presence of Reduced Adams' Catalyst—50 ml. of water and 100 mg. of Adams' catalyst were shaken with hydrogen for 20 minutes. The catalyst was removed by centrifugation and diluted to 50 ml. with an acetate-veronal buffer solution of pH 8.66. To this mixture there was then added 0.500 gm. of free glucosamine and quickly mixed. A 1 ml. portion was immediately removed for sugar estimation and the remainder of the mixture then shaken with hydrogen at atmospheric pressure, in a jacketed vessel cooled with water at a temperature of 20–21°.

A 1 ml. portion was removed at regular intervals for sugar estimation and these values and the value of k are given below. In the calculation of k the concentration of sugar at the end of the 1st hour was used as the initial concentration (a).

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OXIDATION AND HYDROLYSIS OF POLYGALACTURONIDE METHYL ESTER TO LEVO-TARTARIC ACID

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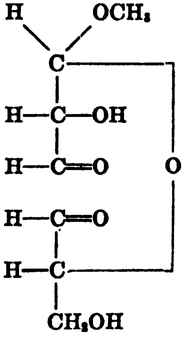
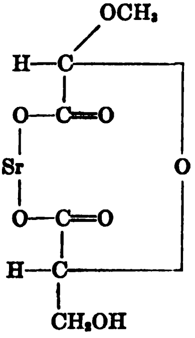
(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(Received for publication, June 28, 1937)

Malaprade[1] introduced periodic acid for the purpose of dissociating the carbon chain of polyhydric glycols. Criegee,[2] a few years later, introduced lead tetraacetate—which generally acts similarly to periodic acid—as an oxidant, particularly for the purpose of recognizing the presence of two adjacent terminal hydroxyl groups in glycosides. Fleury and Lange,[3] at the same time, recognized that periodic acid acts only in the presence of two adjacent hydroxyl groups. Stimulated by the discrepancies in the results obtained by the action of lead tetraacetate and of periodic acid on sugars, Karrer and Pfaehler[4] investigated the



(*a*)



(*b*)

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[1] Malaprade, L., *Bull. Soc. chim.*, **43**, 683 (1928); **1**, 833 (1934).

[2] Criegee, R., *Ber. chem. Ges.*, **64**, 260 (1931).

[3] Fleury, P., and Lange, J., *Compt. rend. Acad.*, **195**, 1395 (1932); *J. pharm. et chim.*, **17**, 196 (1933).

[4] Karrer, P., and Pfaehler, K., *Helv. chim. acta*, **17**, 766 (1934).

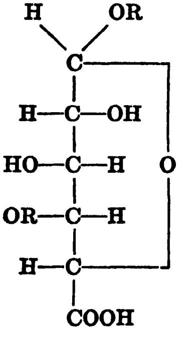
details of the mechanism of the action of periodic acid on methylglucosides and came to the conclusion that formation of formaldehyde during oxidation with periodic acid is preceded by the intermediate formation of an acetal of the structure shown in (*a*).

More recently Jackson and Hudson[5] made a more comprehensive study of the action of periodic acid on glycosides. They oxidized Karrer's dialdehyde into a dicarboxylic acid which was isolated by them in the form of a strontium salt of the composition shown in (*b*).

On further oxidation with subsequent hydrolysis, this product yielded oxalic and glyceric acid. The author offered these findings as direct evidence of the pyranoid structure of the more common glycosides.

Stimulated by the recent applications of periodic acid, particularly those of Jackson and Hudson, we subjected a polygalacturonide prepared by the method of Morell, Baur, and Link[6] to the action of periodic acid. Following the experience of Jackson and Hudson, the oxidation with periodic acid was followed by oxidation with bromine and by hydrolysis. From the product of hydrolysis levo-tartaric acid was isolated in the form of its acid potassium salt.

This finding indicates that the glycosidic units of the polygalacturonate can have only one of the following structures.



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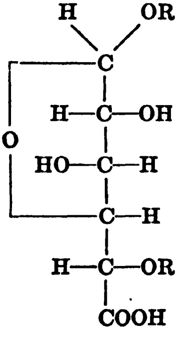

II

(R = galacturonide residue)

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[5] Jackson, E. L., and Hudson, C. S., *J. Am. Chem. Soc.*, **58**, 378 (1936); **59**, 994 (1937).

[6] Morell, S., Baur, L., and Link, K. P., *J. Biol. Chem.*, **105**, 1 (1934). Baur, L., and Link, K. P., *J. Biol. Chem.*, **109**, 293 (1935).

Taking the case of pyranoside structure, had R been in position (3), then the substance would not be oxidized by periodic acid; on the other hand, if R was in position (2), the molecule should have been ruptured in two 3-carbon fragments.

In the case of furanoid structure, only when R is in position (5) could the substance be oxidized by periodic acid, for only then does it contain two adjacent unsubstituted hydroxyl groups.

Thus it may be concluded that the hydroxyl groups of carbon atoms (4) and (5) are engaged in the ring closure and in the condensation of the galacturonic acid residues. The special function of each hydroxyl group remains to be established.

EXPERIMENTAL

Oxidation and Hydrolysis of Polygalacturonide Methyl Ester to Levo-Tartaric Acid—Polygalacturonide methyl ester[6] (2.6 gm.) was dissolved in 100 cc. of water. To this was added a solution of 15.8 gm. (6.0 moles) of crystalline periodic acid[7] (H_5IO_6) in 100 cc. of water, and the whole allowed to stand at room temperature overnight, during which time a light orange color developed.

To this solution were added 12 gm. of strontium carbonate in small portions with stirring to neutralize the mineral acids. Carbon dioxide was evolved and the precipitate, consisting of strontium iodate, strontium periodate, and unchanged strontium carbonate, which settled out was removed by filtration. The filtrate was oxidized by adding 5 cc. of bromine and 15 gm. of strontium carbonate with mechanical stirring for 24 hours at room temperature, at the end of which time the excess bromine was removed by aeration. This solution gives no Fehling's reduction unless previously hydrolyzed with acids.

To this mixture 60 cc. of 6 N sulfuric acid (10 to 15 cc. more sulfate ion than required to combine with all the strontium present) was slowly added, and the strontium sulfate removed by filtration. Silver carbonate (about 10 gm.) was now added to the filtrate until a portion tested with saturated silver sulfate solution gave no precipitate. By this procedure were removed all the bromine ions introduced during the bromine oxidation, and the iodate and periodate ions that had escaped precipitation with strontium.

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[7] Manufactured by G. Frederick Smith Chemical Company, Columbus, Ohio.

After the precipitated silver salts were filtered, the filtrate was treated with hydrogen sulfide to free the solution from silver ions, aerated, and filtered. This yielded a colorless filtrate that still contained sufficient sulfuric acid to effect hydrolysis of the glycosidic bonds present in the oxidized polygalacturonide. Accordingly, the solution was maintained at the boiling point under a reflux and the hydrolysis followed polarimetrically. At the end of 13 hours the rotation had become constant and the hydrolysis was judged complete.

The sulfate ions were now removed quantitatively with barium hydroxide, giving a solution that was practically free from all inorganic matter. This was concentrated under diminished pressure to a volume of 5 cc. Potassium hydroxide (6.0 N) was now added until the solution was just alkaline to phenolphthalein. Glacial acetic acid (about 4 cc.) was introduced and crystallization of the acid potassium tartrate was induced by gently rubbing the inner walls of the flask with a glass rod, and was completed by cooling in the refrigerator.

The crystals were filtered off, washed successively with 50 per cent ethanol, 100 per cent ethanol, and ether and dried at 78° over sulfuric acid under diminished pressure. Yield, 0.55 gm.

The product was purified by recrystallizing from 8 cc. of boiling water, cooling, filtering, washing, and drying as before. Its identity as pure levo-potassium acid tartrate was established by its analysis and its optical behavior.

6.521 mg. substance: 3.012 mg. K_2SO_4
$C_4H_5O_6K$ (188.14). Calculated, K 20.78; found, K 20.72

Its specific rotation was

$$[\alpha]_D^{28} = \frac{-0.09^\circ \times 100}{2 \times 0.204} = -22^\circ \text{ (in water)}$$

A single drop of concentrated hydrochloric acid was added to this same solution and after equilibrium was reached the specific rotation had changed to

$$[\alpha]_D^{28} = \frac{-0.06^\circ \times 100}{2 \times 0.20} = -15^\circ \text{ (in water containing traces of } K^+, H^+, \text{ and } Cl^-\text{)}$$

Pribram[8] gives $[\alpha]_D^{20} = +21.57°$ ($c = 0.25$ in water) for the *dextro*-potassium acid tartrate and $[\alpha]_D^{20} = +14.96°$ ($c = 0.20$ in water) for *dextro*-tartaric acid.

We wish to thank the Research Department of the California Fruit Growers Exchange, Ontario, California, for their generous gift of citrus pectin which has served as our source of the polygalacturonide methyl ester.

[8] Pribram, R., *Monatsh. Chem.*, **14**, 739 (1893).

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THE RING STRUCTURE OF α-METHYL-*d*-GALACTURONIDE AND ITS DERIVATIVES

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(Received for publication, June 28, 1937)

A knowledge of the ring structure of methylgalacturonide is essential for the study of the details of the structure of all polygalacturonides. Evidence in favor of the pyranoside structure was given by Morell and Link[1] who compared the rates of hydrolysis of α-methyl-*d*-galacturonide and of α-methyl-*d*-galactopyranoside. Niemann and Link[2] synthesized α-methyl-*d*-galacturonide by oxidation of diacetone-*d*-galactose which was found by Levene and Meyer[3] to have the pyranose structure.

These investigations made the pyranose structure of methyl-*d*-galacturonide very probable. However, direct evidence was much desired.

With this aim in view, methyl-*d*-galacturonic ester prepared by the method of Morell and Link[4] was exhaustively methylated by the method of Purdie. The α-methyl-2,3,4-trimethyl-α-galacturonide methyl ester was obtained in beautifully crystalline form in practically quantitative yield.

From the product of oxidation of the α-methyl-2,3,4-trimethyl-*d*-galacturonide methyl ester a substance was isolated having the properties of trimethoxy-*l*-araboglutaric acid which was identified as its dimethylamide. This finding established definitely the pyranoside structure of α-methyl-*d*-galacturonide methyl ester and of the many compounds prepared from it, as can be seen from the accompanying formulæ.

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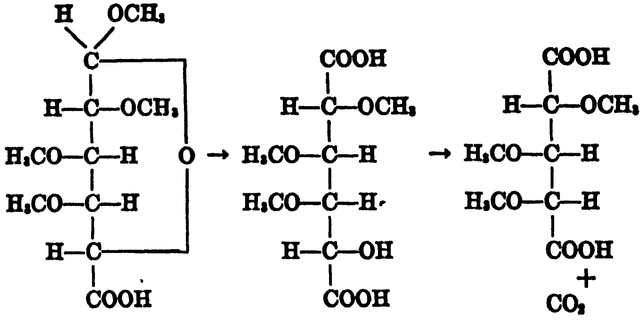
[1] Morell, S., and Link, K. P., *J. Biol. Chem.*, **104**, 183 (1934).

[2] Niemann, C., and Link, K. P., *J. Biol. Chem.*, **104**, 195 (1934).

[3] Levene, P. A., and Meyer, G. M., *J. Biol. Chem.*, **92**, 257 (1931).

[4] Morell, S., and Link, K. P., *J. Biol. Chem.*, **100**, 385 (1933).



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Additional evidence in favor of the pyranoside structure of α-methyl-*d*-galacturonide is found in the behavior of its amide with respect to Weerman and to Hofmann reactions. To our knowledge, no attempt had previously been made to degrade uronic amides by one of these methods. Both of these reactions are general for all α-hydroxy acid amides. Hofmann[5] reports in the case of ethoxyacetamide the formation of a mixed urea derivative yielding on hydrolysis formaldehyde. Haworth, Hirst, and Chamberlain[6] report that 2-methylgluconamide does not undergo the Weerman degradation under the usual conditions. This information reached us after our own experiments had been completed. In experiments performed under the conditions given by Weerman,[7] the only product identified by us from α-methyl-*d*-galacturonamide was the starting material in a very considerable quantity. Under the conditions of the Hofmann reaction, the only product isolated was α-methylgalacturonate dihydrate.

Thus, the attempted degradations failed, but the experience brought forth evidence that the hydroxyl group in position (5) of α-methylgalacturonide is engaged in the ring formation; otherwise the degradations should have been successful.

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[5] Hofmann, A. W., *Ber. chem. Ges.*, **18**, 2734 (1885).

[6] Haworth, W. N., Hirst, E. L., and Chamberlain, K. A., *J. Chem. Soc.*, 795 (1937).

[7] Weerman, R. A., *Rec. trav. chim. Pays-Bas*, **37**, 16 (1918).

EXPERIMENTAL

Anhydrous-α-Methyl-d-Galacturonide Methyl Ester—α-Methyl-*d*-galacturonide methyl ester monohydrate was prepared by the method of Morell and Link.[4]

The monohydrate was dehydrated by drying under reduced pressure over phosphorus pentoxide for 2 days at 78°. It was then dissolved in boiling absolute ethanol (solubility about 3 gm. in 10 cc.) and slowly cooled to 0°. Beautiful flaky crystals appeared which melted sharply at 148° and whose composition agreed with that of anhydrous α-methyl-*d*-galacturonide methyl ester.

4.206 mg. substance: 6.675 mg. CO_2 and 2.415 mg. H_2O

$C_8H_{14}O_7$.	Calculated.	C 43.22, H 6.4
222.11	Found.	" 43.26, " 6.4

α-Methyl-2,3,4-Trimethyl-d-Galacturonide Methyl Ester—Anhydrous α-methyl-*d*-galacturonide methyl ester (8.0 gm.) was placed in a 3-necked flask fitted with an efficient motor stirrer and a reflux condenser. It was dissolved in 35 cc. of anhydrous methanol at 60° and then 35 cc. of methyl iodide were added through the reflux condenser. Vigorous stirring was commenced and continued throughout the reaction. Silver oxide (40.0 gm.) was added portionwise over a period of 8 hours, and the reaction temperature was maintained at 40°. At the end of the 4th hour an additional 20 cc. of methyl iodide were added and at the end of the 6th hour a further 10 cc. of methyl iodide were introduced. (Portionwise addition of the methyl iodide is necessary because of the insolubility of the unmethylated product in this reagent, while portionwise addition of silver oxide is performed so that fresh, unreacted surfaces of this solid will always be available for methylation.[8])

At the end of the 8th hour the stirring was stopped, the reaction mixture allowed to cool to room temperature, and the whole left to stand overnight. 50 cc. of methanol were now added, and the mixture vigorously stirred for a few minutes and then filtered by suction to separate the silver salts from the solution. The silver salts were then extracted four times with 30 cc. portions of boiling methanol. These washings were combined with the main filtrate and the whole was concentrated under diminished pressure to a thick syrup with a bath temperature of less than 40°.

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[8] Hibbert, H., Tipson, R. S., and Brauns, F., *Canad. J. Research*, **4**, 221 (1931).

This syrup was next remethylated twice more, each time with 35 cc. of methyl iodide at 40°, in the absence of extraneous solvent, and 20 gm. of silver oxide added over a period of 6 hours. As before, the methylated product was quantitatively removed from the silver salts with boiling methanol. After the third methylation the entire reaction mass crystallized when the solvents were completely removed.

The substance was recrystallized from ether by dissolving it in 20 cc. or more and evaporating the solvent on the water bath until less than 10 cc. of solvent remained, and cooling in the refrigerator. The average yield is about 8.0 gm., or 85 per cent of the theoretical, but by a remethylation of the mother liquors the yield becomes practically quantitative. The crystals form in solid, massive clumps and melt at 69–70° after one crystallization from ether. They are soluble in water, chloroform, benzene, the lower alcohols, and ether and sparingly soluble in petroleum ether. At the end of the third recrystallization from ether both the melting point and the rotation became constant. Melting point, 70.2–70.3°. Specific rotation

$$[\alpha]_D^{20} = \frac{+4.95° \times 100}{2 \times 1.742} = 142.1° \text{ (in U.S.P. chloroform)}$$

The composition agreed with that of a pentamethylhexuronic acid.

5.193 mg. substance: 9.510 mg. CO_2 and 3.520 mg. H_2O
4.115 " " : 18.505 " AgI
$C_{11}H_{20}O_7$. Calculated. C 49.97, H 7.6, OCH_3 58.72
264.16 Found. " 49.93, " 7.6, " 59.36

Nitric Acid Hydrolysis and Oxidation of α-Methyl-2,3,4-Trimethyl-d-Galacturonide Methyl Ester—α-Methyl-2,3,4-trimethyl-*d*-galacturonide methyl ester (3.00 gm.) was simultaneously hydrolyzed and oxidized with 30 cc. of concentrated (*d* = 1.42) nitric acid. The reaction began at 65° and, as soon as the vigor of the reaction permitted, the temperature was raised to 95° and maintained there for 4 hours. The reaction mixture was then cooled and diluted with 50 cc. of water and transferred to a large Claisen distilling flask. Here the nitric acid was removed by evaporating the solution at 40° to a thick syrup under the vacuum of an efficient water pump, adding 5 to 10 cc. of water, and redissolving the syrup and concentrating again.

After repeating this evaporation cycle about twenty-five times the contents of the flask largely crystallized. The flask was evacuated an additional half hour at 10 mm. pressure and then for 3 more hours at less than 0.1 mm. The product from this treatment was free from nitric acid (nitron test). It was found that the viscous syrup surrounding the crystals was fairly soluble in dry ether, while the crystals did not dissolve appreciably on short contact with ether, so this method was used to effect their separation.

The crystals proved to be trimethoxymucic acid and the syrup was a mixture of oxidation products from which trimethoxy-*l*-araboglutaric acid and trimethoxymucic acid were isolated as their respective crystalline methylamides.

2,3,4-Trimethoxymucic Acid—The crystals obtained from the nitric acid hydrolysis and oxidation of α-methyl-2,3,4-trimethyl-*d*-galacturonide methyl ester were quantitatively removed from the Claisen flask, by dissolving in acetone, transferred to a small Erlenmeyer flask, and the solution evaporated to about 5 cc. To this solution were added 100 cc. of ether and the solution placed in the refrigerator for 4 hours. At the end of this time well formed crystal rosettes had appeared. These were removed from the mother liquor, washed with ether, and when dry weighed 1.26 gm. and melted at 100–101°.

An additional 0.54 gm. was obtained from the mother liquor. After two more recrystallizations from acetone-ether, the melting point remained unchanged at 100–101° and the rotation was

$$[\alpha]_D^{23} = \frac{+2.28^\circ \times 100}{2 \times 2.712} = +42.0^\circ \text{ (in acetone)}$$

Its composition corresponds to that of a trimethoxymucic acid.

4.002 mg. substance: 6.306 mg. CO_2 and 2.280 mg. H_2O
3.420 " " : 24.43 cc. 0.01 N $Na_2S_2O_3$
$C_9H_{16}O_8$. Calculated. C 42.84, H 6.4, OCH_3 36.91
252.12 Found. " 42.96, " 6.4, " 36.92

Dimethyl Ester of 2,3,4-Trimethoxymucic Acid—2,3,4-Trimethoxymucic acid (1.2 gm.) was dissolved in 30 cc. of absolute methanol containing 3 per cent hydrogen chloride and gently refluxed overnight. The hydrogen chloride was removed with silver carbonate and the filtrate evaporated under reduced pressure to a solid crystalline mass. This was dissolved in an excess of ether, filtered, and concentrated to about 15 cc. Crystal plates formed and crystallization was completed in the refrigerator. Yield, 0.97 gm. A second crop of crystals weighing 0.30 gm. was obtained from the mother liquor.

The substance melts sharply at 100.5°. As this is practically the same melting point as that of the acid before esterification, a mixed melting point was made. That the two are entirely different was shown by the fact that the mixture melted at 79–82°. The non-identity of the two substances is substantiated by the elementary analysis and by the rotation. After two recrystallizations from ether the specific rotation was

$$[\alpha]_D^{24} = \frac{+1.28^\circ \times 100}{2 \times 2.217} = +28.9^\circ \text{ (in water)}$$

The composition corresponded to that of the dimethyl ester of a trimethoxymucic acid.

4.792 mg. substance: 8.290 mg. CO_2 and 3.100 mg. H_2O
3.189 " " : 13.305 " AgI
$C_{11}H_{20}O_8$. Calculated. C 47.12, H 7.2, OCH_3 55.37
280.16 Found. " 47.17, " 7.2, " 55.07

Dimethylamide of 2,3,4-Trimethoxymucic Acid—Dimethyl ester of 2,3,4-trimethoxymucic acid (0.25 gm.) was dissolved in 6 cc. of a concentrated solution of methylamine in dry methanol and kept at room temperature for 2 days. The solution was then concentrated to a solid crystalline mass under reduced pressure. It was recrystallized by dissolving it in 5 cc. of methanol and then adding 25 cc. of ethyl acetate. This mixture was evaporated to a volume of 15 to 18 cc. On cooling, clusters of long, stout needles formed. The product was filtered, washed with ethyl acetate, then ether, and dried. Yield, 0.24 gm. (practically quantitative).

The substance melted sharply at 207° and further recrystallization did not raise the melting point. The specific rotation was

$$[\alpha]_D^{23} = \frac{+0.41^\circ \times 100}{2 \times 1.630} = +12.6^\circ \text{ (in methanol)}$$

The analysis indicated that the compound is the dimethylamide of a trimethoxymucic acid.

3.809 mg. substance: 6.610 mg. CO_2 and 2.705 mg. H_2O
4.198 " " : 0.374 cc. N_2 (27° and 756 mm.)
$C_{11}H_{22}O_6N_2$. Calculated. C 47.45, H 8.0, N 10.07
278.19 Found. " 47.32, " 8.0, " 10.08

The diamide of 2,3,4-trimethoxymucic acid was also prepared, but it was found very unsuitable as a derivative for purposes of characterization.

Trimethoxy-l-Araboglutaric Acid from the Oxidation of α-Methyl-2,3,4-Trimethyl-d-Galacturonide Methyl Ester—The non-crystalline fraction remaining after the removal of 2,3,4-trimethoxymucic acid from the mixture obtained by the nitric acid oxidation of α-methyl-2,3,4-trimethyl-*d*-galacturonide methyl ester, was evaporated under reduced pressure to free it from ether, and then dry methanol was added and subsequently removed under reduced pressure four times to eliminate traces of water.

The resulting syrup was dissolved in 20 cc. of dry methanol containing 3 per cent of dry hydrogen chloride and allowed to reflux overnight. The hydrogen chloride was then removed with silver carbonate and the methanol solution concentrated to a thin syrup. This was transferred to a microstill and distilled very slowly at 0.2 mm., the product being divided into four arbitrary fractions: A, 0.3 gm. distilling at a bath temperature of 100–120°; B, 0.4 gm., from 120–140°; C, 0.2 gm., from 140–150°; and a small still residue, D, remaining in the flask. All four fractions were undoubtedly mixtures, since there was no noticeable change in the rate of distillation as the temperature was gradually raised. The first three fractions were converted separately into the methylamides by the procedure recorded for the formation of 2,3,4-trimethoxymucic acid dimethylamide.

Fractions A and C yielded no pure crystalline chemical individual substances. Fraction A yielded a syrup and 0.13 gm. of a crystal mixture that contained both needles and more compact polyhedra and that melted at from 143–150°. After repeated recrystallizations from ethyl acetate and from methanol-ethyl acetate, two fractions were obtained, one melting at 151–161° and the other at 145–153°. Under the microscope both were still seen to be mixtures. Fraction C involved similar difficulties.

We were more successful with Fraction B in that we were able to isolate from it two pure substances: the dimethylamide of 2,3,4-trimethoxymucic acid and the dimethylamide of trimethoxy-*l*-araboglutaric acid. When a methanol-ethyl acetate solution of the methylamides of Fraction B was allowed to crystallize, 0.12 gm. of impure dimethylamide of 2,3,4-trimethoxymucic acid (m.p. 193–197°) separated. The mother liquor on further concentration (in effect, removing the methanol) and cooling yielded 0.13 gm. of impure dimethylamide of trimethoxyglutaric acid, m.p. 162–168°.

The dimethylamide of 2,3,4-trimethoxymucic acid was pure after two recrystallizations from methanol-ethyl acetate and melted at 206–207°. The mixed melting point with a pure authentic sample prepared directly from the crystalline methyl ester showed no depression in the melting point. The specific rotation was

$$[\alpha]_D^{25} = \frac{+0.21° \times 100}{1 \times 1.668} = +12.6° \text{ (in methanol)}$$

and the analysis substantiated the identity.

3.318 mg. substance: 0.296 cc. N_2 (28° and 759 mm.)
$C_{11}H_{22}O_6N_2$ (278.19). Calculated, N 10.07; found, N 10.09

The dimethylamide of trimethoxy-*l*-araboglutaric acid was pure after three recrystallizations from ethyl acetate and melted at 171–172°. A mixed melting point made with an authentic specimen (prepared by Dr. J. Compton in this laboratory) showed no depression. The specific rotation was

$$[\alpha]_D^{25} = \frac{+0.70° \times 100}{1 \times 1.163} = +60.2° \text{ (in water)}$$

which compares favorably with $[\alpha]_D^{18} = +59.9°$ and m.p. 172° reported by Haworth, Hirst, and Jones.[9]

The elementary analysis corresponds to that of the dimethylamide of trimethoxyglutaric acid.

5.313 mg. substance: 9.400 mg. CO_2 and 3.800 mg. H_2O
3.990 " " : 0.397 cc. N_2 (26° at 760 mm.)

$C_{10}H_{20}O_5N_2$.	Calculated.	C 48.35,	H 8.1,	N 11.29
248.18	Found.	" 48.24,	" 8.0,	" 11.36

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[9] Haworth, W. N., Hirst, E. L., and Jones, D. I., *J. Chem. Soc.*, 2428 (1927).

α-Methyl-d-Galacturonamide—Hydrated α-methyl-*d*-galacturonide methyl ester (14.0 gm.) was dissolved in 20 cc. of dry methanol at 50° and the solution cooled to 20°. To this were added 100 cc. of a saturated solution of ammonia in dry methanol at 0° and the solution kept in the refrigerator overnight. A considerable portion of the product crystallized and was collected by filtering. A second crop of crystals was obtained by evaporating the filtrate to a volume of 50 cc. under reduced pressure, leaving in the refrigerator overnight, and then filtering the crystals that had formed. Total yield, 11.8 gm. (97 per cent of the theoretical). This was recrystallized by dissolving in 20 cc. of warm water, slowly adding 150 cc. of warm absolute ethanol with stirring, and then cooling in the refrigerator. M.p. 221–223°. After two more recrystallizations from water-ethanol, as above, the melting point and rotation were constant. M.p. 225–226°. The specific rotation was

$$[\alpha]_D^{23} = \frac{+3.69° \times 100}{2 \times 1.451} = +127.2° \text{ (in water)}$$

It had the composition of α-methylgalacturonamide.

6.402 mg. substance: 0.378 cc. N_2 (25.5° at 762 mm.)
3.301 " " : 9.38 " 0.01 N $Na_2S_2O_3$
$C_7H_{13}O_6N$. Calculated. N 6.76, OCH_3 14.98
207.11 Found. " 6.77, " 14.69

Attempted "Weerman Degradation" of α-Methyl-d-Galacturonamide—The essential points in the method of Weerman[7] were followed. The reaction was very slow both in the cold and at room temperatures, but as the reactants were slowly heated to 60–65° the sodium hypochlorite was rapidly consumed, and after 30 minutes at that temperature it had disappeared entirely (as was shown by a negative test with starch-iodide paper). We were not able to isolate a degradation product, but we did succeed in recovering considerable quantities of a crystalline product which proved to be unchanged starting material.

Attempted "Hofmann Bromamide Degradation"[10] *of α-Methyl-d-Galacturonamide*—α-Methyl-*d*-galacturonamide (2.0 gm.) was dissolved in 5 cc. of water and 0.53 cc. (1.1 molecules) of bromine added and slowly warmed on the water bath. By the time the temperature had reached 60° the bromine had entirely dissolved.

[10] Hofmann, A. W., *Ber. chem. Ges.*, 15, 762 (1882).

A warm solution of 8.0 gm. of hydrated barium hydroxide in 40 cc. of water was then added dropwise and the following changes noted: the deep brown solution became yellow and a white precipitate of barium carbonate appeared. The reaction mixture was maintained at 60–70° for 2 hours, cooled to 15°, and then sulfuric acid added until the solution was just neutral to Congo red. Carbon dioxide was evolved and barium sulfate precipitated.

The precipitate was removed by centrifuging, and the resulting clear solution was freed in turn from bromine, silver, and barium by treating respectively with silver carbonate, hydrogen sulfide, and sulfuric acid, yielding a solution that was practically free from all inorganic matter. Upon evaporation of this solution under reduced pressure a considerable amount of crystalline material was obtained. This, on recrystallization from water-ethanol, yielded 0.9 gm. of crystal plates melting at 108–110° and after three more recrystallizations from the same solvents melting at 110–112°. It had the following composition.

5.514 mg. substance: 6.985 mg. CO_2 and 3.215 mg. H_2O

Halogen and nitrogen were both absent.

$C_7H_{16}O_9$ (244.12). Calculated, C 34.41, H 6.6; found, C 34.54, H 6.5

The specific rotation was

$$[\alpha]_D^{27} = \frac{+2.18^\circ \times 100}{1 \times 1.669} = +130.6^\circ \text{ (in water)}$$

The melting point, analysis, and specific rotation prove the product to be α-methyl-*d*-galacturonide dihydrate, reported by Niemann and Link[2] as having a melting point of 111.0–111.5° and $[\alpha]_D^{20} = +129.0^\circ \pm 1.0^\circ$ ($c = 1.3$ in water).

We wish to thank the Research Department of the California Fruit Growers Exchange, Ontario, California, for their generous gift of citrus pectin which has served as our source of galacturonic acid.

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CONVERSION OF URONIC ACIDS INTO CORRESPONDING HEXOSES

I. Conversion of 2,3,4-Trimethyl-α-Methyl-*d*-Galacturonide Methyl Ester into 2,3,4-Trimethyl-α-Methyl-*d*-Galactoside

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The advancement of our knowledge of the structure of polyuronides is very slow as compared with the progress made in the chemistry of polysaccharides. This slow progress is due to the fact that the terminal carboxyl group renders the molecule of both free and combined uronic acids very labile. It was natural therefore to search for methods for conversion of uronic acids into the corresponding hexoses, by reduction of the carboxyl group, or into pentoses, by the elimination of the carboxyl group.

In a previous communication[1] there has been reported the failure to remove the —COOH group from α-methyl-*d*-galacturonide methyl ester by either the Weerman or the Hofmann reactions. It was now attempted to accomplish a similar task by conversion of 2,3,4-trimethyl-α-methyl-*d*-galacturonide methyl ester into 2,3,4-trimethyl-α-methyl-*d*-galacturononitrile with subsequent elimination of the nitrile group. Three methods—those of Wohl, of Hockett, and of Zemplen—were made use of, all without success.

These methods having failed, an attempt was made to convert 2,3,4-trimethyl-α-methyl-*d*-galacturonide methyl ester into 2,3,4-trimethyl-α-methyl-*d*-galactoside. This task has been accomplished successfully by a series of transformations indicated in formulæ (I) to (VI). The structure of substance (VI) has been identified by hydrolysis to a trimethyl-*d*-galactose having properties identical with those of the *2,3,4-trimethyl-d-galactose* prepared by Onuki[2] through the series of transformations indicated by formulæ (VII) to (XI).

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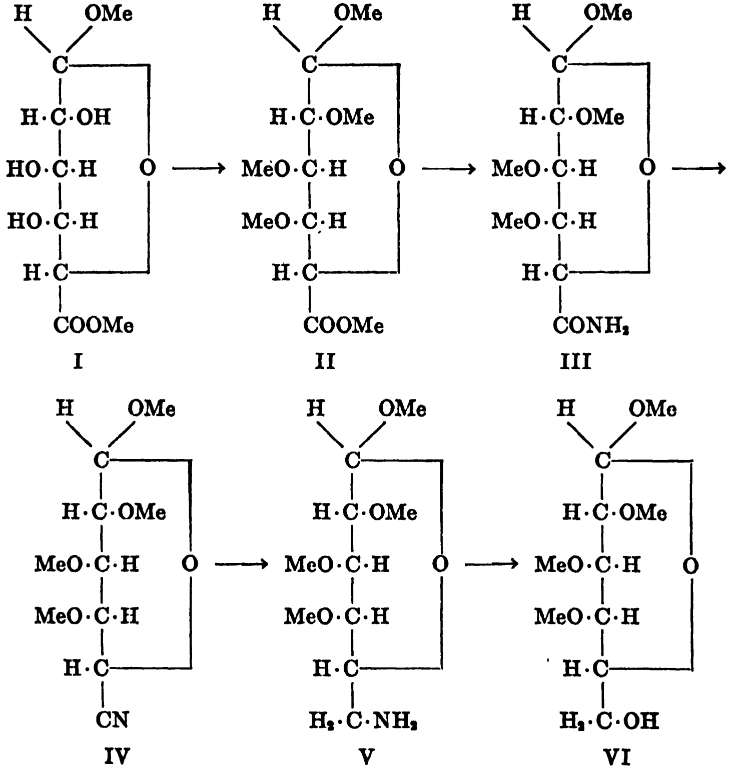
[1] Levene, P. A., and Kreider, L. C., *J. Biol. Chem.*, **120**, 597 (1937).

Further work along these lines as well as on catalytic reduction the $COOCH_3$ group is in progress. Since the date of submission of this manuscript, we have accomplished this task with the collaboration of Dr. R. S. Tipson.

EXPERIMENTAL

Attempted Wohl Degradation[3] of 2,3,4-Trimethyl-α-Methyl-d-Galacturononitrile—2,3,4-Trimethyl-α-methyl-*d*-galacturononitrile



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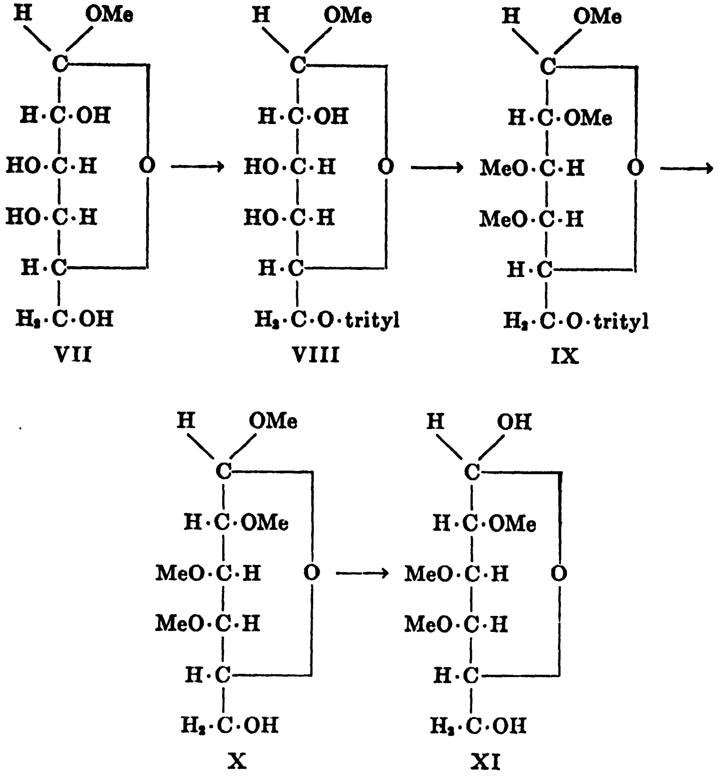
[2] Onuki, M., *Bull. Inst. Physic. and Chem. Research*, Tokyo, **12**, 614 (1933).

[3] Wohl, A., *Ber. chem. Ges.*, **26**, 730 (1893). Wohl, A., and List, E., *Ber. chem. Ges.*, **30**, 3101 (1897).



(1.0 gm.) was dissolved in 20 cc. of absolute methanol and to this was added a solution of 1.0 gm. of silver nitrate dissolved in 10 cc. of concentrated ammonium hydroxide and the whole kept at 25° for 40 hours.

Most of the ammonia was now removed by passing air through the solution. This was then diluted to 100 cc. with water and the silver quantitatively removed with hydrogen sulfide. The silver sulfide was filtered off and the filtrate freed of hydrogen sulfide by aeration. The solution was extracted with 150 cc. of chloroform used in small portions and the chloroform extract washed with water, dried with drierite, and evaporated to a thin syrup under reduced pressure. The last traces of chloroform were removed by thrice adding and evaporating 10 cc. portions of absolute methanol. At this point thin prisms appeared and they were allowed to increase overnight in the cold. It was found that the crystals were quite insoluble in cold ether, while the syrup in which they were embedded was soluble, so advantage was taken of this to separate the crystals. They were recrystallized from ether and melted at 182–183°. The composition of this compound corresponded to a tetramethyl hexuronothioamide.

4.616 mg. substance: 7.695 mg. CO_2 and 3.087 mg. H_2O
4.430 " " : 0.198 cc. N_2 (27° and 755 mm.)
4.985 " " : 17.500 mg. AgI
4.715 " " : 4.270 " $BaSO_4$
$C_{10}H_{19}O_5NS$. Calculated. C 45.25, H 7.2, N 5.28, S 12.09, OCH_3 46.79
265.22 Found. " 45.45, " 7.5, " 5.05, " 12.44, " 46.35

The substance is soluble in chloroform or water, moderately soluble in acetone, less soluble in ethanol, and quite insoluble in ether.

What probably happened was that the Wohl degradation did not take place at all, or at least only to a minor extent, so that the excess of the hydrogen sulfide (that was introduced to remove the silver ions) added to the nitrile to form a thioamide. This reaction appears to be quite general and is known to be catalyzed by ammonia and the amines.[4] In our case ammonia was still present when the hydrogen sulfide was added. Thus the product we obtained must be *2,3,4-trimethyl-α-methyl-d-galacturonothioamide.*

To test this further the same product was prepared in good yield by the action of hydrogen sulfide on an ammoniacal methyl alcoholic solution of 2,3,4-trimethyl-α-methyl-*d*-galacturononitrile.

Attempted Hockett Degradation[5] *of 2,3,4-Trimethyl-α-Methyl-d-Galacturononitrile*—2,3,4-Trimethyl-α-methyl-*d*-galacturononitrile (1.0 gm.) was added to 35 cc. of concentrated ammonium hydroxide and then warmed on a water bath under the hood for 3 hours with occasional shaking, solution being effected at the end of the 1st hour. It was removed from the water bath and concentrated to dryness under diminished pressure. This procedure was repeated thrice.

The last traces of ammonia and water were removed by twice dissolving the resultant crystals in 5 cc. of absolute ethanol and evaporating to dryness (crystallization) under reduced pressure. The water-free crystals were then dissolved in 5 cc. of boiling absolute ethanol; the solution was evaporated to about half of this volume, cooled to 40°, and 50 cc. of pentane slowly added. Crystallization was spontaneous and was complete after standing for 3 hours in the refrigerator. Yield, 0.83 gm. of needle-like crystals that melt at 152–153°. This material, on recrystallization, melted at 153.0–153.5°. The specific rotation was

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[4] Kindler, K., *Ann. Chem.*, **431**, 187 (1923).

[5] Hockett, R. C., *J. Am. Chem. Soc.*, **57**, 2265 (1936).

$$[\alpha]_D^{23} = \frac{+5.30° \times 100}{2 \times 2.182} = +121.4° \text{ (in U.S.P. chloroform)}$$

The mixed melting point with an authentic specimen of 2,3,4-trimethyl-α-methyl-*d*-galacturonamide was unchanged at 153.0–153.5°. The rotation of the same authentic specimen was $[\alpha]_D^{27} =$ +121.5° (c = 2.07 in chloroform).

Attempted Zemplen Degradation[6] *of 2,3,4-Trimethyl-α-Methyl-d-Galacturononitrile*—2,3,4-Trimethyl-α-methyl-*d*-galacturononitrile (5.6 gm.) was dissolved in 25 cc. of chloroform at 0°. To this was added a solution of 1.5 gm. of sodium in 50 cc. of absolute methanol at 0°. This reaction mixture was allowed to warm to room temperature and left to stand overnight, during which time a precipitate appeared. This was dissolved by adding 40 cc. of water, and then carbon dioxide was slowly passed through the mixture for 3 hours.

The chloroform layer was removed and the aqueous layer was extracted with four 30 cc. portions of chloroform. The combined chloroform solutions were washed twice with 30 cc. portions of water, dried over calcium chloride, and then concentrated to a thick syrup under reduced pressure (30° bath temperature). This syrup was dissolved in ether, and when the ether was evaporated under diminished pressure the whole crystallized. Weight 5.2 gm.

This material was recrystallized from 4 cc. of ether and yielded 4.6 gm. of product melting at 58–60°. Purification is difficult, as the product is very soluble in all the common solvents: water, methanol, ethanol, acetone, ether, benzene, chloroform, or pentane. It is not very soluble in cold heptane, but it crystallizes from it poorly. The substance may be distilled at a bath temperature below 140° at 0.2 mm. The distillate crystallizes readily, but these crystals melt less sharply and at a lower temperature than before distillation. The substance was purified by repeated recrystallization from ether, and after ten recrystallizations the melting point and rotation became constant. M.p. 65.0–65.5°. The specific rotation was

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[6] Zemplen, G., and Kiss, D., *Ber. chem. Ges.*, 60, 165 (1927).

$$[\alpha]_D^{24} = \frac{+6.70° \times 100}{2 \times 1.728} = +194° \text{ (in U.S.P. chloroform)}$$

4.902 mg. substance: 9.100 mg. CO_2 and 3.500 mg. H_2O
8.068 " " : 0.355 cc. N_2 (26° and 766 mm.)
3.111 " " : 13.990 mg. AgI
$C_{11}H_{21}O_6N$. Calculated. C 50.16, H 8.0, N 5.32, OCH_3 58.94
263.17 Found. " 50.62, " 8.0, " 5.06, " 59.35

From this it appears most probable that the constituent atoms of methanol reacted with the nitrile group[7] to give *2,3,4-trimethyl-α-methyl-d-galacturonic imino acid methyl ester.* The large yield makes it improbable that any Zemplen degradation took place.

2,3,4-Trimethyl-α-Methyl-d-Galacturonamide—2,3,4-Trimethyl-α-methyl-*d*-galacturonide methyl ester (8.0 gm.) was dissolved in 4 cc. of warm absolute ethanol. This solution was cooled and to it were added 100 cc. of a saturated solution (at 0°) of dry ammonia in absolute ethanol and then left in the refrigerator for 40 hours.

The mixture was now warmed on the water bath to expel most of the unreacted ammonia, and then the remainder of the ammonia and the solvent were removed under reduced pressure at 35°. The product crystallized in the flask. It was recrystallized by dissolving in 10 cc. of absolute ethanol, evaporating on the water bath to about two-thirds of that volume, and then adding 100 cc. of pentane to the solution. Fine, needle-like crystals appeared immediately and crystallization was completed in the refrigerator. Yield, 5.8 gm. (77 per cent of the theoretical). M.p. of crude material, 148–150°. After two more recrystallizations from ethanol-pentane the melting point and the rotation were constant. M.p. 153.0–153.5°.

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[7] Pinner, A., and Klein, F., *Ber. chem. Ges.*, 10, 1889 (1877).

It had the following specific rotation.

$$[\alpha]_D^{27} = \frac{+5.43° \times 100}{2 \times 2.235} = +121.5° \text{ (in U.S.P. chloroform)}$$

The analysis of this compound corresponded with that of a tetramethyl hexuronamide.

6.295 mg. substance: 0.319 cc. N_2 (26.5° and 761 mm.)
4.542 " " : 17.185 mg. AgI
$C_{10}H_{19}O_6$. Calculated. N 5.62, OCH_3 49.81
249.16 Found. " 5.78, " 49.94

The substance is soluble in cold water, methanol, ethanol, chloroform, acetone, or ethyl acetate. It is sparingly soluble in ether and insoluble in petroleum ether.

2,3,4-Trimethyl-α-Methyl-d-Galacturononitrile—2,3,4-Trimethyl-α-methyl-*d*-galacturonamide (6.0 gm.) was refluxed with 48 cc. of thionyl chloride for 2 hours with the careful exclusion of all moisture. An amber color developed. The thionyl chloride was then removed under reduced pressure; when this was accomplished the entire mass crystallized.

It was now dissolved in 25 cc. of chloroform and 25 cc. of heptane were added and the solvents removed under diminished pressure. The treatment with the chloroform and heptane was repeated, the object being the quantitative removal of the thionyl chloride. The crystalline product was dissolved in 30 cc. of boiling absolute ethanol, treated with norit, filtered, and placed in the refrigerator overnight. The yield was 3.7 gm. (66 per cent of the theoretical) of massive polyhedric crystals melting at 155–156°. It may be recrystallized from absolute ethanol, chloroform-heptane, or ethanol-heptane. After two recrystallizations the melting point became constant at 156–157° and the specific rotation was

$$[\alpha]_D^{23} = \frac{+5.23° \times 100}{2 \times 1.472} = +177.6° \text{ (in U.S.P. chloroform)}$$

Its composition agreed with that of tetramethyl hexurononitrile.

4.102 mg. substance: 7.785 mg. CO_2 and 2.625 mg. H_2O
7.905 " " : 0.423 cc. N_2 (26° and 769 mm.)
$C_{10}H_{17}O_5N$. Calculated. C 51.92, H 7.4, N 6.06
231.14 Found. " 51.75, " 7.2, " 6.18

2,3,4-Trimethyl-6-Amino-α-Methyl-d-Galactoside — 2,3,4-Trimethyl-α-methyl-*d*-galacturononitrile (4.0 gm.) was dissolved in 100 cc. of absolute methanol, Raney's catalyst (2.0 gm.) added, and then subjected to a hydrogen atmosphere at a pressure just slightly in excess of 1 atmosphere. Shaking was begun and in the first 15 minutes 2 molecular equivalents of hydrogen were absorbed. The reaction was continued for another 30 minutes but less than 0.02 molecular equivalent was absorbed in that time, so the reaction was judged complete.

The catalyst was removed by centrifuging and washing twice with methanol. The alcoholic solution was then concentrated under diminished pressure, to a thin, colorless syrup and transferred to a distilling flask. The product distilled at 93–95° at 0.15 mm. (bath temperature, 115°). Yield, 2.95 gm. (72 per cent of the theoretical); $n_D^{25} = 1.4651$.

To check the homogeneity of the product it was redistilled at 25 mm. pressure and boiled at 172° (bath temperature, 185°). The first drop had a refractive index of $n_D^{25} = 1.4651$, the bulk of the distillate, $n_D^{25} = 1.4652$, and the last drop, $n_D^{25} = 1.4652$. Its specific rotation was

$$[\alpha]_D^{25} = \frac{+4.40° \times 100}{2 \times 1.364} = +161.3° \text{ (in U.S.P. chloroform)}$$

The composition agreed with that of a trimethylaminomethylhexoside.

5.643 mg. substance: 10.595 mg. CO_2 and 4.540 mg. H_2O
4.010 " " : 0.214 cc. N_2 (26° and 753 mm.)
$C_{10}H_{21}O_5N$. Calculated. C 51.03, H 9.0, N 5.96
235.17 Found. " 51.20, " 9.0, " 6.03

Preparation of 2,3,4-Trimethyl-α-Methyl-d-Galactoside—2,3,4-Trimethyl-6-amino-α-methyl-*d*-galactoside (1.7 gm.) was dissolved in 50 cc. of 30 per cent aqueous potassium nitrite solution. To this were added 15 cc. of glacial acetic acid, together with 3 drops of octanol to reduce foaming, and the whole stirred vigorously for 30 minutes. After that time a microdetermination of amino nitrogen by Van Slyke's method was made, which showed the reaction to be practically complete. The stirring was continued during a further 30 minutes and then the solution was rendered neutral (about pH 6) by means of potassium hydroxide.

This solution was then extracted with four portions of chloroform (50 cc. each), the chloroform extract washed with 50 cc. of water, dried over calcium chloride, filtered, and evaporated to a thick syrup under reduced pressure. At this point a slight but sharp odor was noticed, so the syrup was dried under a high vacuum over phosphorus pentoxide and potassium hydroxide for 20 hours.

The syrup then was odorless and was transferred to a microstill and distilled very slowly at a pressure of 0.05 mm. and a bath temperature of 120–135°, yielding 0.8 gm. of a clear, viscous liquid, having $n_D^{25} = 1.4608$ and the following specific rotation.

$$[\alpha]_D^{28} = \frac{+1.85^\circ \times 100}{1 \times 1.399} = +132.2^\circ \text{ (in methanol)}$$

The substance had the following composition.

4.690 mg. substance: 8.605 mg. CO_2 and 3.590 mg. H_2O.

$C_{10}H_{20}O_6$.	Calculated.	C 50.81, H 8.6
236.16	Found.	" 50.00, " 8.6

Onuki[2] gives $[\alpha]_D^{18} = +112.50^\circ$ (in water) for 2,3,4-trimethyl-α-methyl-*d*-galactoside and describes it as a viscous liquid. The constants presented here are given with some reserve because of the low figure for the percentage of carbon. Owing to the small amount of the material available, it was deemed advisable to hydrolyze this glycoside and to attempt to prepare from it the crystalline 2,3,4-trimethyl-*d*-galactose for the purpose of characterization.

Preparation of 2,3,4-Trimethyl-d-Galactose—2,3,4-Trimethyl-α-methyl-*d*-galactoside (0.7 gm.) was dissolved in 20 cc. of 4 per cent aqueous hydrochloric acid and heated at 95–98° under a reflux condenser for 2 hours. The solution was then cooled to room temperature, the free hydrochloric acid removed with silver carbonate, the silver ions precipitated as sulfide, and the mixture filtered. The slightly colored filtrate was boiled with norit for 10 minutes, filtered, and the colorless filtrate concentrated under reduced pressure to a clear, thick syrup which was left overnight in a desiccator at 1 mm. pressure over phosphorus pentoxide and potassium hydroxide.

The syrup was dissolved in ethyl acetate and pentane was added to turbidity. On slow concentration under reduced pressure the whole crystallized to a mass of thin, long needles. These were recrystallized once from ethyl acetate-pentane and once from ethyl acetate alone. The melting point was 75–80° (not sharp) and the specific rotation was

$$[\alpha]_D^{27} = \frac{+1.65^\circ \times 100}{1 \times 1.099} = +150.2^\circ$$

initially in water, changing slowly and finally becoming constant the 2nd day at +121.0°.

The substance had the following composition.

5.094 mg. substance: 8.998 mg. CO_2, 3.700 mg. H_2O, and 0.085 mg. ash
$C_9H_{18}O_6$. Calculated. C 48.62, H 8.2
222.14 Found. " 48.16, " 8.2, ash 1.66
Calculated as ash-free. " 48.97, " 8.3

2,3,4-Trimethyl-*d*-galactose is variously reported by Onuki as melting at 84°,[8] 85° (sinters at 80°),[9] 85°,[2, 10] and $[\alpha]_D^{21} = +154.07^\circ \xrightarrow{\text{30 hours}} +122.02^\circ$ (in water)[10] and $[\alpha]_D^{20} = +151.6^\circ \xrightarrow{\text{24 hours}} +120.83^\circ$ (in water).[2]

We wish to thank the Research Department of the California Fruit Growers Exchange, Ontario, California, for their generous gift of citrus pectin which has served as our source of galacturonic acid.

[8] Onuki, M., *J. Agric. Chem. Soc. Japan,* **9**, 90 (1933).
[9] Onuki, M., *J. Agric. Chem. Soc. Japan,* **9**, 214 (1933).
[10] Onuki, M., *Proc. Imp. Acad. Tokyo,* **8**, 496 (1933).

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THE STRUCTURE OF MONOACETONE *d*-XYLULOSE

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The two ketopentoses (xylulose and ribulose) share with the two aldotetroses (erythrose and threose) the distinction of being the only unsubstituted true sugars in which the maximum size of the oxygen ring system (if any) is limited to 5 atoms (furanose).

In a former communication[1] we described the preparation of monoacetone *d*-xylulose and pointed out that, since it is nonreducing to boiling Fehling's solution and displays no mutarotation in water, the ketonic group must be linked with the acetone residue, necessitating the formulation of the sugar residue as a ring structure. The furanose ring was suggested, but there was no proof that a smaller ring system might not be present.

Prince and Reichstein[2] have recently oxidized monoacetone *d*-xylulose, obtaining the monoacetone derivative of a monobasic acid which they have named monoacetone *d*-xylosonic acid.

They assume, without evidence, that it has the structure (II) (and therefore assign to monoacetone *d*-xylulose the structure (I)). Actually, the possibility of the substance having either structure (III) or (IV) cannot reasonably be excluded without definite chemical proof.

From the practical standpoint, xylulose is of interest for at least three reasons. In the first place, *l*-xylulose is the pentose isolated from urine, in cases of pentosuria, by Levene and La Forge.[3] Secondly, *l*-xylulose is intimately related to *l*-xylosone, from which vitamin C (the lactone of 3-keto-*l*-gulonic (or *l*-talonic) acid) has been synthesized.[4] Thirdly, it has been shown that, unlike *d*-xylose, *d*-xylulose is readily converted to glycogen in the animal body.[5]

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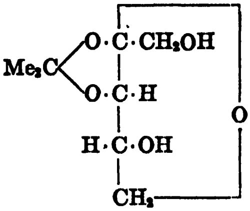
[1] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **106**, 603 (1934).

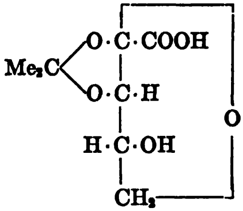
[2] Prince, R., and Reichstein, T., *Helv. chim. acta*, **20**, 101 (1937).

[3] Levene, P. A., and La Forge, F. B., *J. Biol. Chem.*, **18**, 319 (1914).

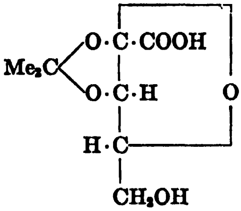
We are also interested in monoacetone *d*-xylulose as a possible starting material for the synthesis of thiomethyl *d*-xylulose, for comparison with the thiomethyl ketopentose obtainable from the adenine thiomethylpentoside present in yeast.



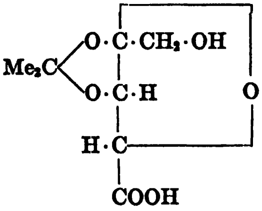
I



II



III



IV

In order to gain some insight into the structure of monoacetone *d*-xylulose it was decided to examine the behavior of its ditosyl ester towards sodium iodide dissolved in acetone.

It has been shown[6] that a tosyl group attached to the primary alcoholic group in glucose derivatives is readily substituted by iodine on treatment with this reagent at 100° during 2 hours. The applicability of the reaction has since been extended to the aldopentofuranoses by Levene and Raymond,[7] but they found that more prolonged treatment (during 6 hours or more) is necessary for such substances and that the rate of reaction is influenced by the presence of other substituents.

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[4] Reichstein, T., *et al.*, *Nature*, **131**, 280 (1933); *Helv. chim. acta*, **16**, 1019 (1933). Haworth, W. N., *et al.*, *J. Chem. Soc.*, 1419 (1933).

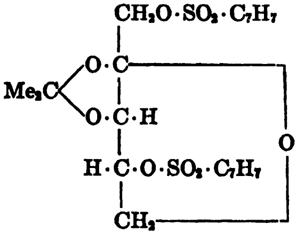
[5] Larson, H. W., Blatherwick, N. R., Bradshaw, P. J., Ewing, M. E., and Sawyer, S. D., *J. Biol. Chem.*, **117**, 719 (1937).

[6] Oldham, J. W. H., and Rutherford, J. K., *J. Am. Chem. Soc.*, **54**, 366 (1932).

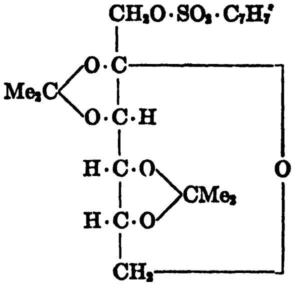
[7] Levene, P. A., and Raymond, A. L., *J. Biol. Chem.*, **102**, 317 (1933).

In the same way it might be expected that a *6-tosyl* ketohexose derivative would react with sodium iodide but, so far as we are aware, no study of the behavior of *1-tosyl* ketohexose derivatives towards this reagent has been made. It is well known that the reactivity of the primary hydroxyl group *adjacent* to the keto group is profoundly influenced by that group and is consequently quite different from that of the primary hydroxyl *remote* from the reducing group.

We have now found that 1-tosyl 2,3,4,5-diacetone fructose is unaffected by sodium iodide in acetone during 2 hours at 100°. *Ditosyl monoacetone d-xylulose* is also unaffected by the same treatment. This evidence indicates that there is no tosyl group attached at carbon atom (5) of the chain, but cannot be regarded as a rigorous proof of the position of the tosyl groups and thence of the structure of the substance. Recourse was therefore had to a study of the methylated derivatives of monoacetone *d*-xylulose.



1,4-Ditosyl monoacetone *d*-xylulose



1-Tosyl β-diacetone *d*-fructose

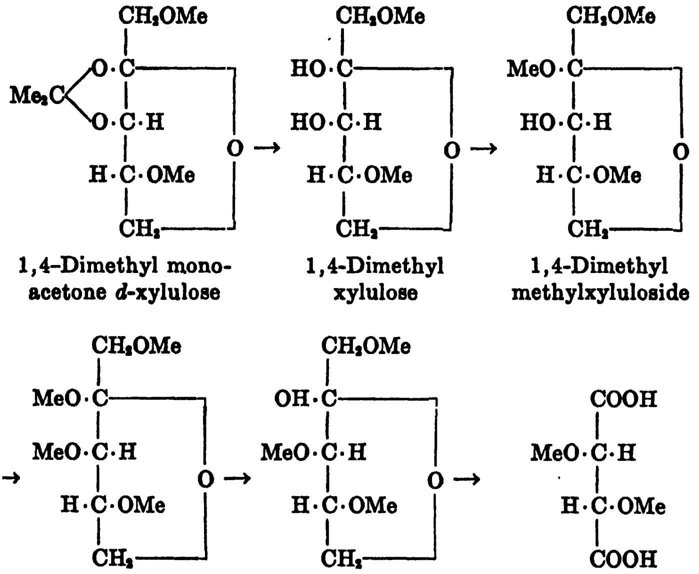
Crystalline monoacetone *d*-xylulose was completely methylated by means of Purdie's reagents to give *dimethyl monoacetone d-xylulose*. On hydrolysis of the acetone residue by means of aqueous oxalic acid solution (0.2 N) a crystalline *dimethyl xylulose*, which was strongly reducing to boiling Fehling's solution, was isolated in practically quantitative yield. The reducing group at position (2) is therefore protected by the acetone residue in the monoacetone derivative, but free in the dimethyl sugar.

The new dimethyl xylulose condensed at room temperature with methyl alcohol (containing hydrogen chloride) with the extreme ease characteristic of furanose sugars. The resulting *dimethyl methylxyluloside* was now methylated by means of Purdie's reagents, forming *trimethyl methylxyluloside.*

The structure of this as *1,3,4-trimethyl methylxylulofuranoside* was established in the following manner. On oxidation with concentrated nitric acid an acid was formed which, on further oxidation by means of acid permanganate, gave crystalline *l*-dimethoxysuccinic acid directly (without intermediate isolation of a dimethyl tetronolactone).

From these results it follows that monoacetone *d*-xylulose is to be designated 2,3-isopropylidene *d*-xylulofuranose.

It is of interest that the isopropylidene group in derivatives of monoacetone xylulose is more resistant towards acid hydrolysis than is the glycosidic methoxyl group in derivatives of methylxyluloside.



1,4-Dimethyl monoacetone *d*-xylulose

1,4-Dimethyl xylulose

1,4-Dimethyl methylxyluloside

1,3,4-Trimethyl methylxyluloside

1,3,4-Trimethyl *d*-xylulose

l-Dimethoxysuccinic acid

EXPERIMENTAL

Preparation of 1,4-Ditosyl Monoacetone d-Xylulose—2.5 gm. of dry, recrystallized monoacetone *d*-xylulose were dissolved in 15 cc. of dry pyridine, 5.5 gm. of tosyl chloride were added, and the mixture shaken until the chloride had dissolved.

After standing overnight at room temperature, with the exclusion of atmospheric moisture, 1 cc. of water was added to the pale brown solution. The resulting solution was kept at room temperature during 30 minutes and the product isolated in the usual manner,[8] giving 6.5 gm. of a colorless gum. The substance was dissolved in 25 cc. of absolute ethanol or methanol (at 40°) and, on cooling to room temperature, it rapidly crystallized.

It had a melting point of 71–73° and the following composition.

4.022 mg. substance: 7.810 mg. CO_2 and 1.895 mg. H_2O
7.122 " " : 6.624 " $BaSO_4$
$C_{22}H_{26}O_9S_2$. Calculated. C 52.98, H 5.3, S 12.87
Found. " 52.95, " 5.3, " 12.78

Its specific rotation was as follows:

$$[\alpha]_D^{25} = \frac{+0.15^\circ \times 100}{2 \times 1.202} = +6.3^\circ \text{ (in acetone)}$$

Action of Sodium Iodide on Ditosyl Monoacetone d-Xylulose—A mixture of 0.5 gm. of dry, recrystallized ditosyl monoacetone *d*-xylulose with 0.6 gm. of sodium iodide was dissolved in 5 cc. of acetone and the solution heated in a sealed tube at 100° during 2 hours. The solution remained colorless and no crystalline material separated out during this treatment.

However, after heating at 100° during 8 hours some crystalline sodium *p*-toluene sulfonate settled out on cooling. The product was isolated in the usual manner, giving a colorless syrup which was dissolved in absolute methanol. On cooling, unchanged starting material (amounting to over 50 per cent of the original weight taken) crystallized out. After filtration, the mother liquor yielded a colorless syrup which contained iodine but which was so contaminated by unchanged starting material that the iodo derivative could not be obtained in crystalline form.

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[8] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **105**, 419 (1934).

Preparation of 1-Tosyl 2,3,4,5-Diacetone d-Fructose[9]—10 gm. of dry, recrystallized β-diacetone fructose were dissolved in 60 cc. of dry pyridine, 8.1 gm. of tosyl chloride were added, and the mixture shaken until the chloride had dissolved.

After standing overnight at room temperature, with the exclusion of atmospheric moisture, 1 cc. of water was added to the pale pinkish yellow solution. The resulting solution was kept at room temperature during 30 minutes and the product then isolated in the usual manner, giving 15.9 gm. of a colorless gum. The substance was dissolved in 25 cc. of absolute ethanol (at 40°) and, on cooling to room temperature, it rapidly crystallized.

It had a melting point of 83° and the following specific rotation.

$$[\alpha]_{\mathrm{D}}^{25} = \frac{-0.76^\circ \times 100}{2 \times 1.470} = -25.9^\circ \text{ (in absolute ethanol)}$$

Its composition was as follows:

4.219 mg. substance: 8.510 mg. CO_2 and 2.395 mg. H_2O
6.235 " " : 3.582 " $BaSO_4$
$C_{19}H_{26}O_8S$. Calculated. C 55.04, H 6.3, S 7.74
Found. " 55.05, " 6.4, " 7.81

Action of Sodium Iodide on 1-Tosyl 2,3,4,5-Diacetone d-Fructose—A mixture of 0.5 gm. of 1-tosyl β-diacetone *d*-fructose with 0.4 gm. of sodium iodide was dissolved in 5 cc. of acetone and the solution heated in a sealed tube at 100° during 2 hours. The solution remained colorless and no crystalline material separated out during this treatment.

Even after heating at 100° during 8 hours the solution remained colorless and no crystalline material separated out on cooling.

Preparation of Dimethyl Monoacetone d-Xylulose—5 gm. of dry, recrystallized monoacetone *d*-xylulose were dissolved in 5 cc. of cold acetone and 50 cc. of methyl iodide were added, the solution remaining clear and colorless. 5 gm. of silver oxide were added and the solution was boiled gently under a reflux, with mechanical stirring. Four further portions (5 gm. each) of silver oxide were added at intervals of 30 minutes and the reaction was allowed to proceed for a total of 5 hours.

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[9] Ohle, H., and Koller, I., *Ber. chem. Ges.*, 57, 1566 (1924).

The product was isolated in the usual manner[8] (dry ether being used as solvent) and remethylated with the same amounts of Purdie's reagents (*without* the addition of acetone).

The resulting syrup (5.6 gm.) was distilled, giving a main fraction (5 gm.) boiling at 47° at 0.1 mm. (bath temperature, 51°) which had $n_D^{25} = 1.4371$. A second fraction (0.3 gm.), which was not quite fully methylated, had $n_D^{25} = 1.4390$. The still residue was of negligible weight.

The main fraction was a colorless, extremely mobile liquid which had the following composition.

5.408 mg. substance: 10.890 mg. CO_2 and 4.090 mg. H_2O
5.952 " " : 12.860 " AgI
$C_{10}H_{18}O_5$. Calculated. C 55.01, H 8.3, OCH_3 28.44
Found. " 54.91, " 8.4, " 28.52

Its specific rotation was as follows:

$$[\alpha]_D^{24} = \frac{-0.33^\circ \times 100}{2 \times 1.308} = -12.6^\circ \text{ (in acetone)}$$

Hydrolysis of Dimethyl Monoacetone d-Xylulose—The course of hydrolysis at 65° of a 2.294 per cent solution of dimethyl monoacetone *d*-xylulose in 0.2 N aqueous oxalic acid (having initial $[\alpha]_D^{25} = +3.2^\circ$) was studied polarimetrically as previously described for monoacetone *d*-xylulose.[10] After 2 hours the substance was only hydrolyzed to the extent of 48 per cent and 10 hours were required for complete hydrolysis, the solution then having $[\alpha]_D^{25} = -17.3^\circ$ (calculated as dimethyl pentose). The intermediate values observed were 71.4 (4 hours), 85.7 (6 hours), and 92.9 per cent (8 hours). No decomposition occurred, since the final rotation remained unchanged after treatment during 24 hours at 65°.

Accordingly, a solution of 5.0 gm. of dimethyl monoacetone *d*-xylulose in 218 cc. of 0.2 N oxalic acid solution was kept in a bath at 65° for at least 10 hours. It was then cooled in ice to room temperature and the product isolated as described for xylulose from monoacetone xylulose,[10] giving 4.0 gm. of a perfectly colorless, thick syrup. The product was dissolved in about 10 cc. of dry ether and pentane was added to faint opalescence, whereupon dimethyl xylulose crystallized in clusters of long, fine needles. In a few minutes it had set to an almost solid mass.

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[10] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **115**, 731 (1936).

The substance was readily distilled (without decomposition) under a high vacuum. It had a boiling point of 88° at 0.2 mm. (bath temperature, 105°) and was collected as a colorless, fairly mobile syrup (having $n_D^{25} = 1.4630$) which crystallized to a solid mass again on nucleation and then had a melting point of 48–49°. It was strongly reducing to boiling Fehling's solution and had a transitory bitter-sweet taste.

Its composition was as follows:

5.102 mg. substance: 8.811 mg. CO_2 and 3.646 mg. H_2O
4.184 " " : 10.950 " AgI
$C_7H_{14}O_5$. Calculated. C 47.16, H 7.9, OCH_3 34.84
Found. " 47.09, " 8.0, " 34.57

It had the following specific rotation.

$$[\alpha]_D^{26} = \frac{-0.54° \times 100}{2 \times 1.010} = -26.7° \text{ (in absolute methanol, 2 min. after admixture)}$$

changing to the constant value $[\alpha]_D^{26} = -16.3°$ (30 minutes).

Preparation of Dimethyl Methylxyluloside—Crystalline dimethyl xylulose condensed rapidly at 26° with methanol containing 1 per cent of hydrogen chloride. The specific rotation at various time intervals was as follows: −11.2° (4 minutes), −9.8° (5 minutes), −14.1° (10 minutes), −15.6° (20 minutes), and −16.6° (30 minutes), constant thereafter.

Accordingly, 2.5 gm. of crystalline dimethyl xylulose were dissolved in 250 cc. of cold methyl alcohol containing 2.5 gm. of hydrogen chloride. The solution was kept at room temperature (26°) during 60 minutes and then dry silver carbonate was added until the solution was neutral to Congo red.

The mixture was filtered and the silver salts extracted three times with boiling dry ether. The filtrate and extracts were combined and evaporated to dryness under diminished pressure. The syrupy product was dissolved in dry ether, a trace of insoluble silver salt filtered off, and the filtrate reevaporated to dryness, giving a perfectly colorless, mobile syrup (weight, 2.7 gm.) which was distilled under a high vacuum.

Three fractions were collected (b.p., 61–64° at 0.25 mm., with bath temperature 70–72°) as colorless, quite mobile liquids with the following properties.

Fraction 1. (0.94 gm.) $n_D^{25} = 1.4479$; $[\alpha]_D^{30} = -18.6°$ (in acetone, $c = 1.24$)
" 2. (1.44 ") " $= 1.4482$; " $= -7.0°$ (" " " $= 1.32$)
" 3. (0.32 ") " $= 1.4485$; " $= +4.3°$ (" " " $= 1.29$)

Though there was no significant difference in boiling point (or refractive index) for each fraction, observation of the specific rotation revealed the partial separation of the α from the β isomer of the glycoside and there is little doubt but that, by repeated fractionation, a pure specimen of each isomer could be obtained.

The composition of each fraction corresponded with that of a dimethyl methylpentoside, *e.g.* the first fraction had the following composition.

5.697 mg. substance: 10.404 mg. CO_2 and 4.290 mg. H_2O
5.511 " " : 20.104 " AgI
$C_8H_{16}O_5$. Calculated. C 49.97, H 8.4, OCH_3 48.45
Found. " 49.80, " 8.4, " 48.15

Methylation of Dimethyl Methylxyluloside—4.0 gm. of dimethyl methylxyluloside were dissolved in 50 cc. of methyl iodide and treated with 25 gm. of silver oxide (added in portions of 5 gm., every 30 minutes), the mixture being boiled gently under a reflux, with mechanical stirring. The reaction was allowed to proceed for a total of 5 hours.

The product was isolated in the usual manner, giving a colorless, very mobile syrup (4.4 gm.) which was distilled under a high vacuum. No attempt was made to separate the α from the β form of the glycoside. It boiled at 52° at 0.25 mm. (bath temperature, 55–56°) and was collected as a colorless, very mobile liquid having $n_D^{25} = 1.4368$ and the following composition.

3.281 mg. substance: 6.300 mg. CO_2 and 2.600 mg. H_2O
3.600 " " : 16.390 " AgI
$C_9H_{18}O_5$. Calculated. C 52.40, H 8.8, OCH_3 60.21
Found. " 52.36, " 8.9, " 60.09

Other workers[11] state that "in view of the highly volatile nature of the trimethyl methylpentosides, the distillations were carried out at a pressure not lower than about 14 mm." In the present case we found that there was no loss of material when the distillation was performed at 0.25 mm., provided that the distillation was slow and the receiver was cooled in ice. A trap, cooled in solid carbon dioxide-chloroform, was connected between the receiver and the high vacuum pump, but no material collected there.

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[11] Haworth, W. N., Hirst, E. L., and Oliver, E., *J. Chem. Soc.*, 1917 (1934).

Hydrolysis of Trimethyl Methylxyluloside—The course of hydrolysis at 65° of a 2.344 per cent solution of trimethyl methylxyluloside in 0.2 N aqueous oxalic acid (having initial $[\alpha]_D^{25} = +1.3°$) was studied polarimetrically, as previously described for the hydrolysis of monoacetone *d*-xylulose.[10] After 30 minutes, hydrolysis was practically complete. The course of the hydrolysis was as follows: 37.8 (5 minutes), 70.8 (15 minutes), 84.1 (20 minutes), 93.0 (25 minutes), 98.7 (30 minutes), and 100 per cent (60 minutes). No decomposition occurred, since the final rotation ($[\alpha]_D^{25} = -14.4°$, calculated as trimethyl pentose) remained unchanged after treatment during 18 hours at 65°.

Accordingly, a solution of 1.17 gm. of trimethyl methylxyluloside in 50 cc. of 0.2 N oxalic acid solution was kept in a bath at 65° for 60 minutes. It was then cooled in ice to room temperature, and the product isolated in the usual manner and distilled under a high vacuum. It had a boiling point of 64° at 0.25 mm. (bath temperature, 72°) and was collected as a colorless, quite mobile liquid having $n_D^{25} = 1.4458$, which was strongly reducing to boiling Fehling's solution.

Its composition was as follows:

3.623 mg. substance: 6.667 mg. CO_2 and 2.695 mg. H_2O
4.302 " " : 40.447 cc. 0.01 N thiosulfate
$C_8H_{16}O_5$. Calculated. C 49.97, H 8.4, OCH_3 48.45
Found. " 50.18, " 8.3, " 48.38

The distilled, syrupy substance had the following specific rotation.

$$[\alpha]_D^{25} = \frac{-0.34° \times 100}{2 \times 1.216} = -14.0° \text{ (in absolute methanol)}$$

Oxidation of Trimethyl Xylulose with Nitric Acid—1 gm. of trimethyl xylulose (or 1.07 gm. of trimethyl methylxyluloside) was dissolved in 10 cc. of concentrated nitric acid ($d = 1.42$) at 25°

and the temperature raised until, at 59°, a vigorous action ensued. The temperature was maintained at 59–60° during 15 minutes and then gradually raised until, after a further 75 minutes, it had reached 95°.

The solution was now cooled and evaporated to dryness under diminished pressure at 50° with a distilling flask provided with a trap (to prevent loss by splashing) at the point of attachment of the side arm. A portion (10 cc.) of water was added and the solution evaporated to dryness. The process of adding water in portions of 10 cc. and evaporating to dryness after each addition was repeated until a sample of the distillate gave no precipitate on addition of a few drops of a 10 per cent solution of nitron in 5 per cent aqueous acetic acid, together with a few drops of dilute sulfuric acid, and allowing to stand. In this manner, the whole of the nitric acid was removed in some 4 hours (as compared with the customary period, reported in the literature, of several days).

The product, consisting of a colorless syrup containing a small amount of crystalline material, was dried by adding benzene and evaporating to dryness, the procedure being repeated several times. It was now dissolved in dry ether, a trace of insoluble material filtered off, and the filtrate evaporated to dryness in a tared dish. It was dried overnight, over phosphorus pentoxide and potassium hydroxide, in a vacuum desiccator at room temperature (27°) and 0.05 mm. The colorless syrup, containing a few colorless, rectangularly shaped crystals, weighed 0.89 gm. (theoretical yield, 0.93 gm.).

Esterification and Methylation of Dimethyl Xylosonic Acid—The crude dimethyl xylosonic acid (0.89 gm.) was esterified in the following manner. It was first freed from traces of water by dissolving in absolute methanol and evaporating the solution to dryness, this procedure being repeated three times.

The syrup was then dissolved in 25 cc. of absolute methanol containing 3 per cent of dry hydrogen chloride and the solution boiled, under a reflux condenser closed by a calcium chloride tube, during 6.5 hours.

The solution was now cooled in ice and rendered neutral to Congo red by the addition of finely powdered silver carbonate. The mixture was filtered and the silver salts well washed with dry ether. The filtrate and washings were combined and evaporated to dryness under diminished pressure. The product was dissolved in dry ether, filtered from a trace of insoluble impurity, and the filtrate evaporated to dryness, giving a pale yellow syrup (weight, 0.8 gm.) which was distilled at high vacuum.

Only one fraction was collected. This boiled at 89–90° at 0.20 mm. (bath temperature, 99–100°) and weighed 0.73 gm. It had $n_D^{25} = 1.4424$ and was reducing to boiling Fehling's solution.

It had the following composition.

4.422 mg. substance: 7.694 mg. CO_2 and 2.600 mg. H_2O
4.892 " " : 46.45 cc. 0.01 N thiosulfate

(*a*) $C_8H_{14}O_6$.	Calculated.	C 46.58,	H 6.9,	OCH_3 45.16
(*b*) $C_9H_{16}O_6$.	"	" 49.06,	" 7.3,	" 56.37
(*c*) $C_8H_{14}O_6$.	"	" 46.58,	" 6.9,	" 60.21
	Found.	" 47.44,	" 6.6,	" 49.10

The product was apparently a mixture of the methyl ester of dimethyl xylosonic acid (*a*) with its "methylglycoside" (*b*), but, as shown later, it also contained a little dimethyl *l*-dimethoxysuccinate (*c*).

It was therefore subjected to complete methylation by means of Purdie's reagents. The product was isolated in the usual manner and distilled at high vacuum. The first fraction (0.2 gm.) boiling at 70–72° at 0.1 mm. (bath temperature, 84–85°) had $n_D^{25} = 1.4371$. It was dissolved in 5 cc. of absolute methanol saturated with dry ammonia. After standing in the refrigerator during 2 days, star-like clusters of long, colorless needles separated. The product was filtered off, washed with ethyl acetate, and dried. It had a melting point of 270° (with previous darkening[12] at 250°) and its composition corresponded with that of the diamide of a dimethoxysuccinic acid.

4.603 mg. substance: 6.907 mg. CO_2 and 2.910 mg. H_2O
5.389 " " : 0.745 cc. N_2 (755 mm. at 28°)

$C_6H_{12}O_4N_2$.	Calculated.	C 40.88,	H 6.9,	N 15.91
	Found.	" 40.91,	" 7.1,	" 15.56

Oxidative Degradation of Dimethyl Xylosonic Acid by Means of Acid Permanganate—The method employed was essentially that of Haworth and Learner[18] except that, in order to avoid the presence of any manganese salt in the final product, the use of carbon dioxide as a neutralizing agent was eliminated.

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[12] Haworth, W. N., and Jones, D. I., *J. Chem. Soc.*, 2349 (1927).

Syrupy crude dimethyl xylosonic acid (0.8 gm.) was dissolved in 22 cc. of 0.5 N sulfuric acid and the solution diluted to 50 cc. with distilled water. An aqueous solution of barium permanganate (approximately 4 per cent) was now slowly added, with shaking, to the solution at room temperature until manganese oxides began to be precipitated.

A cold, saturated aqueous solution of barium hydroxide was then added until the solution was alkaline to thymolphthalein and further addition of barium hydroxide to a centrifuged test portion gave no brown precipitate.

The mixture was centrifuged and the precipitate washed by centrifuging with several changes of water. The solution and washings were united and N sulfuric acid was added dropwise until only a trace of barium remained in solution. The mixture was centrifuged and the precipitate washed with water.

The solution and washings were combined and evaporated to dryness under diminished pressure. The product was dissolved in dry ether, a trace of insoluble barium salt filtered off, and the filtrate evaporated to dryness, giving a solid mass of colorless crystals (weight, 0.5 gm.). It was recrystallized by dissolving in the minimum of boiling, dry ether, adding an equal volume of pentane, and cooling. On nucleating and stirring it separated in beautiful colorless crystals having a melting point of 151° and the the following specific rotation.

$$[\alpha]_D^{28} = \frac{-1.55^\circ \times 100}{2 \times 1.126} = -68.8^\circ \text{ (in water)}$$

It had the following composition.

4.912 mg. substance: 7.305 mg. CO_2 and 2.688 mg. H_2O
4.200 " " : 27.98 cc. 0.01 N thiosulfate
$C_6H_{10}O_6$. Calculated. C 40.43, H 5.7, OCH_3 34.84
Found. " 40.55, " 6.1, " 34.44

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[18] Haworth, W. N., and Learner, A., *J. Chem. Soc.*, 619 (1928). Avery, J., Haworth, W. N., and Hirst, E. L., *J. Chem. Soc.*, 2308 (1927).

[Reprinted from THE JOURNAL OF BIOLOGICAL CHEMISTRY, September, 1937, Vol. 120, No. 2, pp. 621–623]

A NOTE ON THE ACRIDINE SALTS OF "YEAST" AND "MUSCLE" ADENYLIC ACIDS

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In the course of an investigation dealing with the preparation of 5-phosphoadenosine from adenosine, occasion was taken to prepare the acridine salt of natural "muscle" adenylic acid, with a view to utilizing the same method later for the precipitation of the synthetic substance.

The product obtained from the natural acid had a composition quite different from that recorded by Wagner-Juaregg.[1] Whereas he gives the formula $C_{13}H_9N \cdot C_{10}H_{14}O_7N_5P \cdot H_2O$, the acridine salt now described had a composition agreeing with the formula $C_{13}H_9N \cdot 2C_{10}H_{14}O_7N_5P$.

A similar product, prepared by combining "yeast" adenylic acid with acridine, also had a composition indicating the formula $C_{13}H_9N \cdot 2C_{10}H_{14}O_7N_5P$.

EXPERIMENTAL

Acridine Salt of Authentic "Muscle" Adenylic Acid—The acridine salt was prepared as described by Wagner-Juaregg.[1] After being recrystallized twice from water, it was dried in a vacuum desiccator over phosphorus pentoxide, at 0.1 mm. and room temperature, during 2 days.

The lemon-yellow product had a melting point of 217–218°, with previous slight darkening in color. Its composition was as follows:

4.626 mg. substance: 7.785 mg. CO_2 and 1.805 mg. H_2O
4.310 " " : 0.652 cc. N_2 (762 mm. at 25°)
4.510 " " : 21.895 mg. ammonium phosphomolybdate

$C_{13}H_9N \cdot 2C_{10}H_{14}O_7N_5P$.	Calculated.	C 45.34,	H 4.3,	N 17.64,	P 7.10
	Found.	" 45.89,	" 4.4,	" 17.38,	" 7.05

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[1] Wagner-Juaregg, T., *Z. physiol. Chem.*, **239**, 188 (1936).

Wagner-Juaregg contended that the substance had the formula

$C_{13}H_9N \cdot C_{10}H_{14}O_7N_5P \cdot H_2O$. Calculated. C 50.71, H 4.63, N 15.44, P 5.70
$C_{13}H_9N \cdot C_{10}H_{14}O_7N_5P$. " " 52.45, " 4.41, " 15.97, " 5.90

The dried acridine salt had the following specific rotation

$$[\alpha]_D^{25} = \frac{-0.25° \times 100}{2 \times 0.538} = -23.2° \text{ (in 10\% HCl)}$$

5 minutes after admixture. Recalculated as free adenylic acid, this gives a value of $[\alpha]_D^{25} = -29.2°$, which supports our formulation of the substance. Embden and Schmidt[2] state that "muscle" adenylic acid has $[\alpha]_D^{20} = -26.0°$ (in 10 per cent hydrochloric acid), but the observed data which they record give the value $[\alpha]_D^{20} = -31.0° (\pm 1.0°)$.

After 14 days at room temperature, this solution had $[\alpha]_D^{25} = +13.9°$, which, recalculated as 5-phosphoribose, gives a value of $[\alpha]_D^{25} = +26.5°$, affording further verification of the above formulation. From the data recorded by Levene and Stiller,[3] the specific rotation of 5-phosphoribose is calculated to be $[\alpha]_D^{26} = +25.6°$ to $+26.0°$.

Acridine Salt of Authentic "Yeast" Adenylic Acid—To a solution of 300 mg. of yeast adenylic acid in 17 cc. of boiling distilled water was added a solution of 154.8 mg. of twice recrystallized acridine in 2 cc. of absolute ethanol, giving a deep yellow solution but no crystals. The solution was now evaporated to dryness in a vacuum desiccator, giving a mass of yellow crystals. The product was twice recrystallized, first from 5 cc. of boiling distilled water and then from 6 cc. of the same solvent. The lemon-yellow crystalline product was dried in a vacuum desiccator over phosphorus pentoxide, at 0.1 mm. and room temperature during 3 days.

The dried substance had a melting point of 183–184° (with no previous darkening) and the following composition.

4.511 mg. substance: 7.505 mg. CO_2 and 1.950 mg. H_2O
4.520 " " : 0.688 cc. N_2 (775 mm. at 26°)
3.895 " " : 18.915 mg. ammonium phosphomolybdate
$C_{33}H_{37}O_{14}N_{11}P_2$. Calculated. C 45.34, H 4.3, N 17.64, P 7.10
Found. " 45.37, " 4.8, " 17.71, " 7.05

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[2] Embden, G., and Schmidt, G., *Z. physiol. Chem.*, **181**, 130 (1929).

[3] Levene, P. A., and Stiller, E. T., *J. Biol. Chem.*, **104**, 299 (1934).

Observation of the specific rotation of this preparation afforded confirmation of the above formulation. It had

$$[\alpha]_{\mathrm{D}}^{25} = \frac{-0.30^\circ \times 100}{2 \times 0.524} = -28.6^\circ \text{ (in 10\% HCl)}$$

10 minutes after admixture. Recalculated as free adenylic acid, this gives a value of $[\alpha]_{\mathrm{D}}^{25} = -35.9^\circ$. Embden and Schmidt[8] record $[\alpha]_{\mathrm{D}}^{20} = -36.5^\circ$ for "yeast" adenylic acid in this solvent.

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PHOSPHORYLATION OF MONOACETONE ADENOSINE AND OF DIACETYL ADENOSINE

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(From the Laboratories of The Rockefeller Institute for Medical Research)

(Received for publication, July 7, 1937)

The substances to be described in the present communication have been prepared in the course of work aiming at the partial synthesis of "muscle" adenylic acid starting from adenosine. Since no difficulty had been encountered in the partial synthesis of inosinic acid (hypoxanthine 5-phosphoribofuranoside) from inosine,[1] the task of synthesis of the corresponding adenylic acid centered on the preparation of some adenosine derivative having the amino group of the adenine residue and the hydroxyl groups in positions (2) and (3) of the ribose residue temporarily blocked, so that a phosphoryl group might be introduced into position (5) of the sugar (it having been shown previously[2] that the hydroxyl group at position (4) is engaged by the oxygen ring).

Considerable difficulty was, however, encountered in the preparation of such a substance. Theoretically it should be readily preparable by several procedures. Thus, 5-trityl triacetyl adenosine or 5-trityl tribenzoyl adenosine might be expected, on treatment with very weak acids, to lose only the trityl group leading to a substance of the desired type. Actually, simultaneously with the scission of the trityl group from the terminal hydroxyl, the acetyl or benzoyl group attached to the amino group is removed. However, only the trityl group is split from tritosyl trityl adenosine, giving tritosyl adenosine.

At this stage it seemed feasible to restore the acetyl group to the nitrogen atom by treatment with ketene. We were unable to acetylate the amino group of diacetyl adenosine with this reagent. Similarly, treatment of monoacetone adenosine with dibenzoyl disulfide in ethanol (even during 3 hours at the boiling point) failed to benzoylate the free amino group, a quantitative yield of unchanged starting material being recovered. Thus the amino group of adenosine apparently displays properties more suggestive of an acid amide than of an amine.

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[1] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **111**, 313 (1935).

[2] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **94**, 809 (1931–32).

It seemed possible to take advantage of this property of the amino group in adenosine for the preparation of muscle adenylic acid, since the diphospho-adenosine which should result from the phosphorylation of adenosine substituted in positions (2) and (3) of the ribose residue should lose that phosphoryl group attached to the nitrogen atom more readily than that at position (5) of the sugar. In a certain sense this expectation was realized. Both diphospho-adenosine and adenylic acid have been prepared—neither, however, in absolutely pure form. When more starting material becomes available, final purification of both substances should offer no special difficulties. Incidentally, the properties of the synthetic diphospho-adenosine may serve to test the validity of the existing theories of the structure of the natural diphospho- and triphospho-adenosines.

Experiments are now in progress aiming at the preparation of triacetyl adenosine by cleavage of 5-trityl triacetyl adenosine through catalytic hydrogenation. Attention is also being directed to the synthesis of adenylic acid by a method hitherto not employed for the preparation of the phosphoric esters of sugars.

It must be mentioned that all the derivatives of adenosine used in the course of the present investigation were previously unknown, so that it was necessary to establish their structure.

Structure of Diacetyl Adenosine—Crystalline monotrityl adenosine was prepared by condensing adenosine with triphenylmethyl chloride in the presence of dry pyridine. By analogy with monotrityl uridine, which has been shown[3] to be the 5-trityl ether, it seemed probable that monotrityl adenosine also has the substituent in the terminal position of the sugar chain. Convincing evidence to this effect was lacking, however, particularly in view of the fact that a *di*trityl adenosine is readily obtainable.

Treatment of monotrityl adenosine with *p*-toluene sulfonyl chloride in dry pyridine gave a *tritosyl trityl adenosine*, from which the trityl group was hydrolyzed by means of boiling 80 per cent aqueous acetic acid to give a crystalline *tritosyl adenosine*. Since the tosyl residues in this compound were *not* replaced by iodine on treatment with sodium iodide in acetone (during 2 hours at 100°) it follows that there was no tosyl group at position (5). Monotrityl adenosine is therefore the 5-trityl ether.

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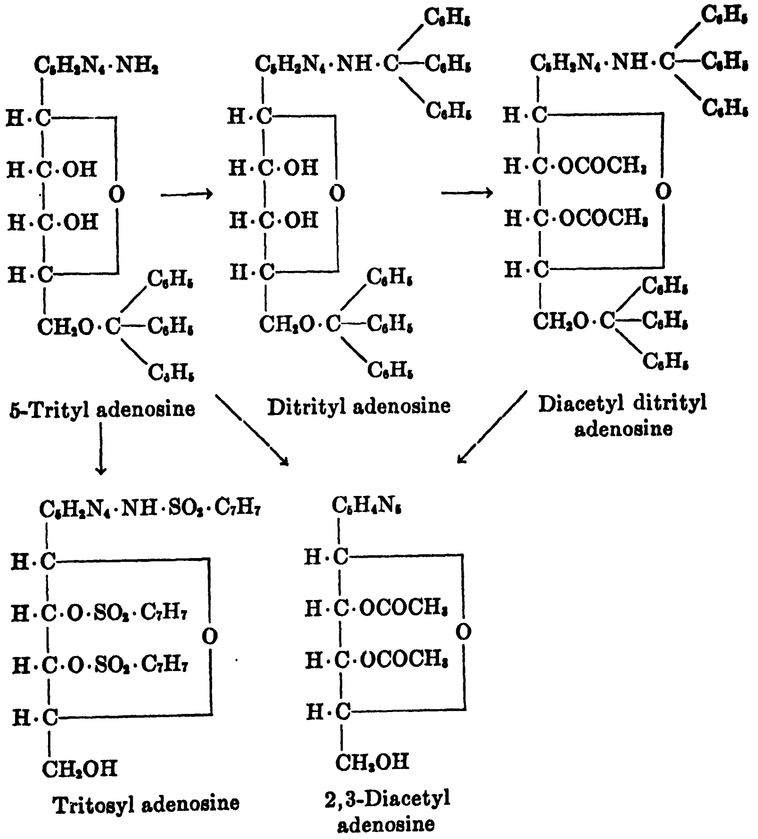
[3] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **105**, 419 (1934).



5-Trityl adenosine

Ditrityl adenosine

Diacetyl ditrityl adenosine

Tritosyl adenosine

2,3-Diacetyl adenosine

The structure of the *ditrityl adenosine* (formed simultaneously with monotrityl adenosine) was established in the following manner. The substance was acetylated to give *diacetyl ditrityl adenosine*. Hydrolysis of this compound by means of boiling

80 per cent aqueous acetic acid gave the *same* diacetyl adenosine (having a free amino group) as was obtained by hydrolysis of *N-acetyl 2,3-diacetyl 5-trityl adenosine* under the same conditions. It follows that the diacetate is *2,3-diacetyl adenosine* and that the ditrityl adenosine is *N-trityl 5-trityl adenine-ribofuranoside.*

This formulation was confirmed by treatment of the diacetyl adenosine with *p*-toluene sulfonyl chloride, giving a ditosyl diacetyl adenosine in which only one tosyl group was substituted by iodine on treatment with sodium iodide.

Similarly, the dibenzoate obtained by hydrolysis of *N-benzoyl 2,3-dibenzoyl 5-trityl adenosine* has the structure of *2,3-dibenzoyl adenosine.*

EXPERIMENTAL

Action of Triphenylmethyl Chloride on Adenosine—Attempts were made to prepare monotrityl adenosine by the method described by Bredereck,[4] but the yield of pure monotrityl ether amounted to only 36 per cent of the theoretical. Bredereck reported a yield of only about 31 per cent. We isolated much material having the properties of a ditrityl adenosine.

Recourse was therefore had to the method successfully used for the preparation of monotrityl thymidine,[5] the yield of monotrityl adenosine being increased to about 50 per cent of the theoretical.

It was observed that adenosine, carefully purified by recrystallization from water as previously described,[1] usually contained a small amount of impurity insoluble in boiling pyridine. It was therefore further purified as follows:

10 gm. of finely powdered adenosine (recrystallized from water and then dried in the vacuum oven at 110° during 48 hours) were suspended in 250 cc. of dry pyridine and the mixture boiled, under a reflux condenser closed by a calcium chloride tube, until most of the adenosine had dissolved.

The undissolved material was then filtered off and the filtrate evaporated to dryness under diminished pressure, giving a crystalline mass which was stirred with a little absolute methanol, and the mixture filtered. The product was washed with dry ether

[4] Bredereck, H., *Z. physiol. Chem.*, **223**, 61 (1934).

[5] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **109**, 623 (1935).

until free from pyridine and again dried in a vacuum oven at 110° during 48 hours.

10 gm. of dry, finely powdered adenosine (purified as above) were suspended in 300 cc. of dry pyridine and the mixture boiled, under a reflux condenser closed by a calcium chloride tube, until all the adenosine had dissolved. The solution was then cooled to room temperature, 11.5 gm. of dry triphenylmethyl chloride were added, and the solution allowed to stand, with the exclusion of atmospheric moisture, during 7 days at room temperature.

The solution, which was still clear and very pale yellow in color, was then poured into 2 liters of filtered ice water, with vigorous stirring. The pale yellow gum which was precipitated changed to a white powder after the mixture was allowed to stand overnight in the refrigerator. The product was filtered off, washed with water until free from pyridine, and dried. The yield of this crude material was about 19 gm.

(*a*) *Monotrityl Adenosine*—The crude product contained, besides monotrityl adenosine, a considerable quantity of *di*trityl adenosine and a small amount of tritanol. It was fractionated in the following manner.

The dry, crude material was dissolved in 150 cc. of boiling dry pyridine and 300 cc. of cold absolute ethanol were added. On cooling and allowing to stand in the refrigerator, 9.2 gm. of monotrityl adenosine were deposited. After washing with a little absolute ethanol and then dry ether, the substance was obtained as pure, colorless crystals having a melting point of 250° (uncorrected). Further small crops of monotrityl adenosine were isolated from the mother liquors (see part (*b*)).

It was soluble in cold pyridine or glacial acetic acid; fairly soluble in cold or hot absolute methanol, absolute ethanol, or acetone; sparingly soluble in cold or hot chloroform or ethyl acetate; very sparingly soluble in cold or hot dry ether, carbon tetrachloride, benzene, pentane, or heptane; and insoluble in cold or hot water.

It had the following composition.

4.706 mg. substance: 11.795 mg. CO_2 and 2.205 mg. H_2O
4.208 " " : 0.510 cc. N_2 (757 mm. at 27°)
$C_{29}H_{27}O_4N_5$. Calculated. C 68.34, H 5.3, N 13.75
Found. " 68.34, " 5.3, " 13.73

Its specific rotation was as follows:

$$[\alpha]_D^{23} = \frac{-0.36° \times 100}{2 \times 1.000} = -18.0° \text{ (in dry pyridine)}$$

Bredereck[4] records for trityl adenosine $[\alpha]_D^{20} = -8.7°$ (in chloroform, $c = 2.069$). We were unable to prepare even a 1 per cent solution of the substance in cold chloroform.

(*b*) *Ditrityl Adenosine*—The pyridine-alcohol mother liquor from the crystalline monotrityl adenosine was evaporated to dryness, giving a pale yellow gum. Weight, about 10 gm. It was obtained as a flaky, glass-like substance by dissolving in acetone and again evaporating to dryness. The product was now treated with 250 cc. of boiling heptane, under a reflux condenser, dissolving all the tritanol and a little of the ditrityl adenosine.

The mixture was filtered while hot and, on cooling, the filtrate deposited crystalline tritanol contaminated with a little ditrityl adenosine.

The material which was insoluble in boiling heptane was dried, finely powdered, and then shaken with 100 cc. of cold acetone. A little insoluble material (monotrityl adenosine) was filtered off, washed with cold acetone, and the combined filtrate and washings evaporated to dryness under diminished pressure.

The glass-like product was dissolved in a little acetone, an equal volume of benzene added, and the solution evaporated to half its volume. An equal volume of benzene was again added and the solution evaporated somewhat until crystallization commenced. A second and a third crop were obtained by evaporating the mother liquor and treating in the same manner.

This ditrityl adenosine was still contaminated with a trace of monotrityl adenosine. It was purified as follows: 7.5 gm. of impure, crystalline ditrityl adenosine were dissolved in 50 cc. of boiling benzene and the hot solution filtered to remove the small amount of insoluble impurity (monotrityl adenosine). On cooling the very pale yellow filtrate, ditrityl adenosine separated immediately in colorless crystals which were filtered off and washed once with benzene, once with a little dry ether, and then dried. The first crop weighed 6.0 gm.

It had a melting point of 200–202° and the following composition.

4.058 mg. substance: 11.390 mg. CO_2 and 2.000 mg. H_2O
4.772 " " : 0.392 cc. N_2 (752 mm. at 24°)
$C_{48}H_{41}O_4N_5$. Calculated. C 76.66, H 5.5, N 9.32
Found. " 76.54, " 5.5, " 9.34

It displayed the following specific rotations.

$$[\alpha]_D^{25} = \frac{-0.33° \times 100}{2 \times 1.064} = -15.5° \text{ (in acetone)}$$

$$[\alpha]_D^{25} = \frac{-0.39° \times 100}{2 \times 1.016} = -19.2° \text{ (in dry pyridine)}$$

It was soluble in cold absolute methanol, absolute ethanol, acetone, chloroform, carbon tetrachloride, pyridine, ethyl acetate, or glacial acetic acid; fairly soluble in cold but soluble in hot benzene; fairly soluble in cold or hot dry ether; sparingly soluble in cold or hot pentane; sparingly soluble in cold but somewhat more soluble in hot heptane; and insoluble in cold or hot water.

Preparation of Tritosyl Monotrityl Adenosine—5 gm. of dry, recrystallized monotrityl adenosine were dissolved in 60 cc. of boiling dry pyridine under a reflux condenser closed by a calcium chloride tube. The solution was cooled to 0° in ice-salt, 6.2 gm. of tosyl chloride were added, and the mixture shaken until the chloride had dissolved. After standing at room temperature during 30 minutes its color had changed to a rich golden yellow and the solution became thick and turbid.

The mixture was kept at room temperature during 3 days, after which time a yellow precipitate had settled and the supernatant liquor was clear and very pale brown in color. The product was isolated in the usual manner,[3] giving a quantitative yield of a colorless, flaky glass having

$$[\alpha]_D^{25} = \frac{-1.16° \times 100}{2 \times 1.014} = -57.2° \text{ (in acetone)}$$

It was soluble in cold acetone, chloroform, benzene, pyridine, ethyl acetate, or glacial acetic acid; only fairly soluble in cold but soluble in hot absolute methanol, absolute ethanol, or carbon tetrachloride; fairly soluble in cold or hot dry ether; insoluble in cold but very sparingly soluble in hot heptane; and insoluble in cold or hot pentane or water.

Its composition was as follows:

3.904 mg. substance: 8.905 mg. CO_2 and 1.750 mg. H_2O
$C_{50}H_{45}O_{10}N_5S_3$. Calculated. C 61.75, H 4.7
Found. " 62.20, " 5.0

Detritylation of Tritosyl Trityl Adenosine—8 gm. of tritosyl trityl adenosine were dissolved in 80 cc. of glacial acetic acid and 20 cc. of water were added. The solution was boiled under a reflux condenser during 30 minutes, cooled, and kept overnight at room temperature. The tritanol (1.3 gm.) which crystallized out was filtered off.

The filtrate was evaporated to dryness under diminished pressure, with addition of toluene and then a mixture of ethanol and benzene, giving a solid crystalline mass. It was dissolved in acetone and, on addition of pentane to faint opalescence, it crystallized.

The substance was recrystallized as follows: 2 gm. were dissolved in 25 cc. of cold acetone, a little charcoal was added, and the mixture filtered. Addition of 18 cc. of pentane gave a faint opalescence and, on stirring, the substance settled out in colorless crystals having a melting point of 195–196° and the following specific rotation.

$$[\alpha]_D^{20} = \frac{-1.97^\circ \times 100}{2 \times 1.044} = -94.4^\circ \text{ (in acetone)}$$

It had the following composition.

4.591 mg. substance: 8.581 mg. CO_2 and 1.835 mg. H_2O
5.705 " " : 0.485 cc. N_2 (748 mm. at 29°)
5.096 " " : 4.835 mg. $BaSO_4$
$C_{31}H_{31}O_{10}N_5S_3$. Calculated. C 51.00, H 4.3, N 9.60, S 13.18
Found. " 50.99, " 4.5, " 9.45, " 13.03

It was soluble in cold absolute methanol, ethanol, acetone, chloroform, pyridine, ethyl acetate, or glacial acetic acid; fairly soluble in cold but somewhat more soluble in hot carbon tetrachloride; fairly soluble in cold and soluble in hot benzene; sparingly soluble in cold or hot dry ether; insoluble in cold but sparingly soluble in hot water; insoluble in cold or hot pentane or heptane.

A mixture of 0.5 gm. of dry, recrystallized tritosyl adenosine with 0.6 gm. of dry sodium iodide was dissolved in 5 cc. of acetone and the solution heated in a sealed tube at 100° during 2 hours. No trace of crystalline material separated out during this treatment.

Preparation of Triacetyl Monotrityl Adenosine—10 gm. of dry, recrystallized monotrityl adenosine were dissolved in 120 cc. of boiling, dry pyridine under a reflux condenser closed by a calcium chloride tube. The solution was cooled to room temperature and 60 cc. of acetic anhydride were added. After standing overnight at room temperature, the solution was poured into 1 liter of ice water with vigorous stirring.

The white powdery precipitate was filtered off, and washed several times with distilled water. The product was purified as previously described for diacetyl trityl uridine,[6] giving a practically quantitative yield of a colorless, flaky glass which was soluble in cold absolute methanol, absolute ethanol, dry ether, acetone, chloroform, carbon tetrachloride, benzene, pyridine, ethyl acetate, or glacial acetic acid; fairly soluble in cold or hot pentane; fairly soluble in cold but quite soluble in hot heptane; and insoluble in cold or hot water.

For analysis, the flaky, glass-like substance was dried in the vacuum oven at 78° during 24 hours. It had the following composition.

4.452 mg. substance: 10.795 mg. CO_2 and 2.110 mg. H_2O
5.780 " " : 0.544 cc. N_2 (755.5 mm. at 24.5°)
6.250 " " : 2.93 " 0.01 N sodium thiosulfate[7]

$C_{35}H_{33}O_7N_5$.	Calculated.	C 66.11,	H 5.3,	N 11.02,	$COCH_3$ 20.32
	Found.	" 66.12,	" 5.3,	" 11.02,	" 20.16

The substance displayed the following specific rotations.

$$[\alpha]_D^{24} = \frac{-0.14° \times 100}{2 \times 1.034} = -6.8° \text{ (in acetone)}$$

$$[\alpha]_D^{24} = \frac{+0.15° \times 100}{2 \times 1.195} = +6.3° \text{ (in absolute methanol)}$$

$$[\alpha]_D^{24} = \frac{+0.29° \times 100}{2 \times 1.036} = +14.0° \text{ (in dry pyridine)}$$

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[6] Levene, P. A., and Tipson, R. S., *J. Biol. Chem.*, **104**, 385 (1934).

[7] Elek, A., and Harte, R. A., *Ind. and Eng. Chem., Anal. Ed.*, **8**, 267 1936).

Preparation of Crystalline Diacetyl Adenosine—Removal of the triphenylmethyl group by means of glacial acetic acid containing hydrogen bromide proved to be too drastic a procedure to employ with this furanoside. The following method was found to be satisfactory.

11 gm. of triacetyl trityl adenosine were dissolved in 320 cc. of glacial acetic acid and 80 cc. of distilled water were added. The resulting clear solution was boiled under a reflux condenser during 30 minutes, cooled in ice to room temperature, and then poured onto ice. Some 450 gm. of sodium bicarbonate, mixed into a paste with water, were now cautiously added until the free acetic acid had been neutralized, sufficient water being added to retain in solution the sodium acetate formed.

The mixture was now extracted three times with chloroform, and the chloroform extracts combined and washed once with dilute sodium bicarbonate solution and once with water. It was later found that, owing to the solubility of adenosine acetate in water, it is advisable to reextract each aqueous layer with chloroform. The chloroform solution was dried with anhydrous sodium sulfate, the mixture filtered, and the filtrate evaporated under diminished pressure to a colorless, crystalline mass weighing about 9.5 gm.

The adenosine derivative was freed from tritanol as follows: The mixture was dissolved in 100 cc. of acetone and pentane (some 75 cc.) was added to faint opalescence. Crystallization commenced immediately and was completed by allowing the mixture to stand overnight in the refrigerator. The crystalline product was filtered off and washed with dry ether to remove traces of tritanol. It was recrystallized by dissolving in acetone and adding pentane to faint opalescence.

It had a melting point of 181–182° and was soluble in cold absolute methanol, absolute ethanol, acetone, chloroform, pyridine, ethyl acetate, glacial acetic acid, or water; fairly soluble in cold or hot carbon tetrachloride or benzene; slightly soluble in cold but fairly soluble in hot dry ether; and insoluble in cold or hot pentane or heptane.

Its composition was as follows:

4.902 mg. substance: 8.605 mg. CO_2 and 2.192 mg. H_2O
4.800 " " : 0.838 cc. N_2 (763 mm. at 25.5°) (Dumas)
21.60 " " : 1.47 " " (762 " " 24°) (Van Slyke)
7.383 " " : 4.174 " 0.01 N sodium thiosulfate

$C_{14}H_{17}O_6N_5$

Calculated. C 47.84, H 4.9, N 19.94, amino N 3.99, $COCH_3$ 24.50
Found. " 47.87, " 5.0, " 20.04, " " 3.80, " 24.31

The substance displayed the following specific rotations.

$$[\alpha]_D^{20} = \frac{-1.64° \times 100}{2 \times 1.042} = -78.7° \text{ (in acetone)}$$

$$[\alpha]_D^{25} = \frac{-0.32° \times 100}{2 \times 0.530} = -30.2° \text{ (in water)}$$

Since the yield of diacetyl adenosine was below the theoretical, the reaction was further investigated, as follows:

12.5 gm. of triacetyl trityl adenosine were dissolved in 400 cc. of 80 per cent aqueous acetic acid and the solution boiled under a reflux during 30 minutes. The solution was then cooled in ice to room temperature and evaporated to dryness under diminished pressure, the bath temperature being kept below 35°. This treatment was without deleterious effect, since the yield of adenosine diacetate was larger than when the acetic acid was removed by neutralization.

During the course of the evaporation a crystalline substance was deposited. Traces of water and acetic acid were removed by adding a little absolute ethanol and toluene and reevaporating to dryness, the procedure being repeated several times.

The crystalline mass was now treated with 100 cc. of cold acetone, but only a part of the material dissolved. The mixture was filtered, the insoluble substance washed with a little acetone, and diacetyl adenosine (5.1 gm.) was isolated from the filtrate as previously described.

The substance (about 0.5 gm.) which was insoluble in acetone was recrystallized as follows: It was dissolved in 200 cc. of boiling water, with the addition of a few drops of glacial acetic acid. The hot solution was filtered and the filtrate allowed to cool. The product, which crystallized readily, had a composition corresponding to that of monoacetyl *adenine*.

4.539 mg. substance: 7.915 mg. CO_2 and 1.602 mg. H_2O
3.700 " " : 1.285 cc. N_2 (755 mm. at 26°)

$C_7H_7ON_5$. Calculated. C 47.43, H 4.0, N 39.55
Found. " 47.55, " 4.0, " 39.38

It had a melting point of 347–348° and, when dissolved in pyridine, was optically inactive under conditions where a specific rotation of ±1° would easily have been detectable.

Preparation of Diacetyl Ditrityl Adenosine—5 gm. of dry, recrystallized ditrityl adenosine were dissolved in 25 cc. of dry pyridine and 20 cc. of acetic anhydride were added. The solution was kept overnight at room temperature and then poured into 500 cc. of filtered ice water with vigorous stirring.

The white, powdery precipitate was filtered off, washed with distilled water until free from pyridine, and then dried in a vacuum desiccator. Yield, quantitative.

It had the following composition.

4.384 mg. substance: 12.005 mg. CO_2 and 2.210 mg. H_2O
5.730 " " : 0.426 cc. N_2 (760.5 mm. at 29°)
$C_{52}H_{45}O_6N_5$. Calculated. C 74.70, H 5.4, N 8.38
Found. " 74.67, " 5.6, " 8.40

Its specific rotation was as follows:

$$[\alpha]_D^{28} = \frac{-0.14^\circ \times 100}{2 \times 1.166} = -6.0^\circ \text{ (in acetone)}$$

It was insoluble in cold or hot water; fairly soluble in cold or hot pentane; fairly soluble in cold but quite soluble in hot heptane; and readily soluble in the following solvents in the cold: absolute methanol, absolute ethanol, dry ether, acetone, chloroform, carbon tetrachloride, benzene, pyridine, ethyl acetate, or glacial acetic acid.

Detritylation of Diacetyl Ditrityl Adenosine—5 gm. of diacetyl ditrityl adenosine were dissolved in 80 cc. of glacial acetic acid and 20 cc. of distilled water were added. The mixture was boiled under a reflux condenser during 30 minutes and then cooled to room temperature. The tritanol (2.1 gm.) which crystallized out was filtered off.

The filtrate was evaporated to dryness under diminished pressure, giving a colorless, crystalline mass (3 gm.), which was freed from tritanol as previously described. The product had the properties previously given for diacetyl adenosine.

Tosylation of Diacetyl Adenosine—2 gm. of dry, recrystallized diacetyl adenosine were dissolved in 20 cc. of dry pyridine, 2.4

gm. of tosyl chloride were added, and the mixture shaken until the chloride had dissolved.

After standing overnight at room temperature, with the exclusion of atmospheric moisture, 0.5 cc. of water was added to the brown solution. The resulting solution was kept at room temperature during 30 minutes and then the product was isolated in the usual manner, giving 2.5 gm. of a colorless, flaky, glassy substance.

It was soluble in cold methanol, acetone, chloroform, pyridine, ethyl acetate, or glacial acetic acid; fairly soluble in cold but soluble in hot absolute ethanol; fairly soluble in cold but somewhat more soluble in hot dry ether, carbon tetrachloride, or benzene; insoluble in cold but very sparingly soluble in hot pentane or water; insoluble in cold or hot heptane.

Its specific rotation was as follows:

$$[\alpha]_D^{28} = \frac{-0.02^\circ \times 100}{2 \times 1.054} = -1.0^\circ \text{ (in acetone)}$$

It contained a little non-ionizable chlorine,[3] indicating the presence of some tosyl diacetyl chloro-adenosine, and had the following composition.

4.700 mg. substance: 0.533 cc. N_2 (750 mm. at 28°)
4.922 " " : 0.280 mg. AgCl
$C_{28}H_{29}O_{10}N_5S_2$. Calculated. N 10.62, Cl 0.00
$C_{21}H_{22}O_7N_5SCl$. " " 13.37, " 6.77
Found. " 12.69, " 1.40

A portion of the substance was treated with an equal weight of dry sodium iodide in acetone in a sealed tube at 100° during 2 hours. The sodium salts which crystallized out were then filtered off, washed with a little acetone, dried, and weighed. Yield, almost quantitative.

Preparation of Tribenzoyl Monotrityl Adenosine—The benzoyl derivative of monotrityl adenosine was investigated, since it was considered possible that the N-benzoyl group might be more stable towards acid hydrolysis than the N-acetyl group.

5 gm. of dry, recrystallized monotrityl adenosine were dissolved in 60 cc. of boiling, dry pyridine under a reflux condenser closed by a calcium chloride tube. The solution was cooled to 0° in

ice-salt and 4.5 cc. of benzoyl chloride were added. After standing at room temperature during 2 days, 1 cc. of water was added and the mixture was kept at room temperature during 30 minutes.

The solution was now poured into 1 liter of ice water with vigorous stirring, and the white, powdery precipitate filtered off and washed several times with distilled water. The product was dissolved in chloroform and the solution shaken with sodium bicarbonate solution and then with water. It was dried by means of anhydrous sodium sulfate, the mixture filtered, and the filtrate evaporated under diminished pressure to a colorless, flaky glass (8 gm.) which was soluble in cold acetone, chloroform, carbon tetrachloride, benzene, pyridine, ethyl acetate, or glacial acetic acid; fairly soluble in cold but soluble in hot absolute methanol or ethanol; fairly soluble in cold or hot dry ether; sparingly soluble in cold but fairly soluble in hot heptane; very sparingly soluble in cold or hot pentane; and insoluble in cold or hot water.

For analysis, the flaky, glass-like substance was dried in the vacuum oven at 78° during 24 hours. It had the following composition.

4.407 mg. substance: 11.802 mg. CO_2 and 1.910 mg. H_2O
5.402 " " : 0.398 cc. N_2 (766.5 mm. at 25.5°)
$C_{50}H_{39}O_7N_5$. Calculated. C 73.05, H 4.8, N 8.53
Found. " 73.02, " 4.8, " 8.49

The substance displayed the following specific rotations.

$$[\alpha]_D^{26} = \frac{-1.34° \times 100}{2 \times 1.012} = -66.2° \text{ (in acetone)}$$

$$[\alpha]_D^{26} = \frac{-0.85° \times 100}{2 \times 1.024} = -41.5° \text{ (in dry pyridine)}$$

Preparation of Crystalline Dibenzoyl Adenosine—7.5 gm. of tribenzoyl trityl adenosine were dissolved in 240 cc. of glacial acetic acid and 60 cc. of water were added. The resulting clear, colorless solution was boiled under a reflux condenser during 30 minutes, cooled in ice to room temperature, and evaporated to dryness under diminished pressure, with alternate additions of absolute ethanol and toluene towards the end of the evaporation.

The crystalline product was dissolved in 25 cc. of boiling acetone. On cooling and stirring, a substance crystallized in long prismatic needles. The crystals were filtered off, washed with a little cold acetone, and dried. Weight, about 1 gm.

This crystalline substance was recrystallized as follows: 1.5 gm. were dissolved in 40 cc. of boiling absolute methanol under a reflux condenser, the hot solution filtered, and the filtrate kept in the refrigerator overnight. The substance, which crystallized in colorless needles (several cm. in length) and which gave no test for pentose with orcinol, gave results on analysis corresponding with those of a monobenzoyl *adenine*.

4.499 mg. substance: 9.975 mg. CO_2 and 1.510 mg. H_2O
4.001 " " : 1.025 cc. N_2 (755 mm. at 26.5°)

$C_{12}H_9ON_5$.	Calculated.	C 60.22,	H 3.8,	N 29.29
	Found.	" 60.46,	" 3.8,	" 29.00

It had a melting point of 237–238° and was soluble in cold pyridine or glacial acetic acid; quite soluble in cold absolute methanol or ethanol; only fairly soluble in cold but soluble in hot acetone, chloroform, or ethyl acetate; sparingly soluble in cold or hot dry ether; very sparingly soluble in cold or hot carbon tetrachloride, benzene, pentane, or heptane; almost insoluble in cold but readily soluble in hot water. It may be recrystallized well from boiling water.

It was optically inactive, in pyridine, under conditions ($c = 1.0$, $l = 2$) where a specific rotation of ±1° would easily have been detectable.

Dibenzoyl adenosine was isolated from the acetone mother liquor (from the crystalline monobenzoyl adenine) in the following manner.

The solution was evaporated to dryness and the product treated with 250 cc. of boiling carbon tetrachloride under a reflux. A part of the material dissolved. The mixture was cooled and, after being kept in the refrigerator overnight, filtered. The filtrate contained tritanol and a little dibenzoyl adenosine.

The material insoluble in carbon tetrachloride was dissolved in acetone and an equal volume of pentane added (to faint opalescence). On standing in the refrigerator, dibenzoyl adenosine slowly crystallized out. It had a melting point of 132–134° and the following specific rotation.

$$[\alpha]_D^{28} = \frac{-2.79° \times 100}{2 \times 1.294} = -107.8° \text{ (in acetone)}$$

It had the following composition.

5.219 mg. substance: 0.661 cc. N_2 (765 mm. at 25.5°)
$C_{24}H_{21}O_6N_5$. Calculated, N 14.74; found, N 14.57

It was soluble in cold absolute methanol, acetone, chloroform, pyridine, ethyl acetate, or glacial acetic acid; fairly soluble in cold but soluble in hot absolute ethanol; fairly soluble in cold or hot dry ether; sparingly soluble in cold but more soluble in hot carbon tetrachloride or benzene; very sparingly soluble in cold or hot pentane; insoluble in cold but very sparingly soluble in hot heptane or water.

Preparation of Crystalline Monoacetone Adenosine—20 gm. of finely powdered adenosine (dried at 120° under diminished pressure during 2 days) were dissolved in a filtered solution of 60 gm. of anhydrous zinc chloride in 600 cc. of acetone and the product isolated as described for monoacetone inosine,[1] except that the final separation of the monoacetone adenosine from barium chloride was accomplished by exhaustively extracting the dry, finely powdered mixture with hot, dry acetone in a Soxhlet apparatus.[8]

The acetone extract was evaporated to dryness under diminished pressure, with the addition of some absolute methanol to reduce the ease of crystallization during the evaporation, giving a colorless, crystalline mass. Yield, 20.7 gm. (90 per cent of the theoretical).

It had a melting point of 200–204° (with slight softening at 190°) and the following composition.

4.792 mg. substance: 8.915 mg. CO_2 and 2.410 mg. H_2O
3.410 " " : 0.686 cc. N_2 (760 mm. at 28.5°)
$C_{13}H_{17}O_4N_5$. Calculated. C 50.78, H 5.6, N 22.80
Found. " 50.74, " 5.6, " 22.83

It displayed the following specific rotations.

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[8] We have recently succeeded in preparing crystalline monoacetone guanosine by the same method.

$[\alpha]_D^{25} = \frac{-1.33° \times 100}{2 \times 1.040} = -63.9°$ (in water; dissolved by warming)

$[\alpha]_D^{25} = \frac{-1.98° \times 100}{2 \times 0.992} = -99.8°$ (in dry pyridine)

$[\alpha]_D^{25} = \frac{-1.06° \times 100}{2 \times 1.010} = -52.5°$ (in 0.1 N aqueous oxalic acid solution; practically unchanged after 18.5 hours at room temperature)

When dissolved in 0.1 N aqueous hydrochloric acid, it had an initial specific rotation of

$$[\alpha]_D^{25} = \frac{-1.66° \times 100}{2 \times 1.536} = -54.0°$$

which changed smoothly to the constant value $[\alpha]_D^{25} = -48.0°$, calculated as adenosine, after 23.5 hours at room temperature. The final value is in agreement with that given by Levene *et al.*[9] for adenosine, namely $[\alpha]_D^{22} = -50.1°$ at pH 1.64.

When dissolved in N hydrochloric acid, hydrolysis of the acetone group is complete in 35 minutes at room temperature. In this solvent, very slow hydrolysis of the adenine residue took place at room temperature as follows: 12.4 (18 hours), 27.9 (42 hours), 54.1 (90.5 hours), 68.1 (116.5 hours), and 99.1 per cent (306 hours).

Phosphorylation Experiments—As may be seen from the following, the phosphorylation product from both monoacetone adenosine and diacetyl adenosine probably is mainly a diphospho derivative. Hence, in order to isolate 5-phospho-adenosine it is necessary to remove the acetone residue, or the acetyl groups, together with the N-phospho group without removing either the purine or the 5-phospho group. The respective stability of the latter two groups in natural "muscle" adenylic acid was therefore first studied under a variety of conditions.

Authentic "muscle" adenylic acid had the following specific rotation in 10 per cent hydrochloric acid.

$[\alpha]_D^{25} = \frac{-0.32° \times 100}{2 \times 0.524} = -30.5°$ (5 min. after admixture)

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[9] Levene, P. A., Simms, H. S., and Bass, L. W., *J. Biol. Chem.*, 70, 243 (1926).

Embden and Schmidt[10] state that the substance has $[\alpha]_D^{20} = -26.0°$ in the above solvent, but we calculate the value $[\alpha]_D^{20} = -31.0°$ ($\pm 1.0°$) from the observed data which they record.

On standing at room temperature, the specific rotation of the above solution (in 10 per cent hydrochloric acid) showed a smooth change as follows: $-27.5°$ (1 hour), $-7.8°$ (24 hours), $+2.9°$ (52 hours), $+8.8°$ (76 hours), $+11.8°$ (96 hours), $+13.7°$ (120 hours), $+15.7°$ (144 hours), and $+17.2°$ (168 and 192 hours). Recalculated as 5-phospho ribose, the final specific rotation is $[\alpha]_D^{25} = +25.9°$. From the data recorded by Levene and Stiller[11] we calculate that free 5-phospho ribose has $[\alpha]_D^{26} = +25.6°$ to $+26.0°$ (in water).

Calculated in terms of the per cent hydrolysis of the *adenine residue*, these results are 7.4 (1 hour), 48.1 (24 hours), 70.3 (52 hours), 82.6 (76 hours), 88.7 (96 hours), 92.7 (120 hours), 96.8 (144 hours), and 100 per cent (168 hours). On the other hand, at the end of 192 hours at room temperature, less than 2 per cent (actually 1.76 per cent) of the *total phosphorus* present had been liberated as inorganic phosphorus by 10 per cent hydrochloric acid.

In less concentrated hydrochloric acid solution, the substance is even more stable. Dissolved in N hydrochloric acid there was no measurable change in specific rotation during 2 days *at room temperature*. For this reason, N hydrochloric acid is preferable to 10 per cent acid (which has hitherto been employed) as a solvent for the determination of the specific rotation of the nucleotide. However, after longer standing at room temperature, hydrolysis of the purine residue by N hydrochloric acid became appreciable: 17.7 (4 days), 21.7 (5 days), 35.5 (11 days), 45.3 (13 days), and 72.9 per cent (27 days). On the other hand, the adenine was completely removed[12] by N hydrochloric acid, *after heating at 100° during 7 minutes*, to give 5-phospho ribose with less than 0.1 per cent of the organic phosphoric acid liberated as inorganic phosphate.

A solution of muscle adenylic acid (0.0868 gm.) in 5 cc. of

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[10] Embden, G., and Schmidt, G., *Z. physiol. Chem.*, **181**, 130 (1929).

[11] Levene, P. A., and Stiller, E. T., *J. Biol. Chem.*, **104**, 299 (1934).

[12] Lohmann, K., *Biochem. Z.*, **233**, 460 (1931).

0.05 N hydrochloric acid, rapidly diluted to 10 cc. with 0.1 N hydrochloric acid, had the following specific rotation.

$$[\alpha]_D^{22} = \frac{-0.71° \times 100}{2 \times 0.868} = -40.9° \text{ (1 hr. after admixture)}$$

After 5 days at room temperature the specific rotation was unchanged and less than 1 per cent of the organic phosphoric acid had been liberated as inorganic phosphate. Muscle inosinic acid is stable under similar conditions.[1]

Phosphorylation of Monoacetone Adenosine—The phosphorylation of monoacetone adenosine was performed exactly as previously described for the preparation of phospho monoacetone inosine.[1] The composition of the product apparently depended on the proportion of phosphorus oxychloride employed and on the length of time during which the reaction was allowed to proceed. Using 1.1 moles of phosphorus oxychloride during 2 hours gave a product in a part of which the amino group had been phosphorylated. Increasing the length of time of reaction and the proportion of the esterifying agent increased the proportion of this N-phospho derivative.

(a) With 1.1 Moles of Phosphorus Oxychloride (2 Hours)—The product was a white powder which had the following composition, after drying at 100°.

5.506 mg. substance: 0.559 cc. N_2 (769.5 mm. at 26°) (Dumas)
44.10 " " : 1.29 " " (769 " " 27°) (Van Slyke)
4.665 " " : 24.800 mg. ammonium phosphomolybdate
39.86 " " : 18.380 " $BaSO_4$
$C_{13}H_{16}O_7N_5PBa$. Calculated. N 13.40, amino N 2.68, P 5.94, Ba 26.29
Found. " 11.33, " " 1.63, " 7.72, " 27.11

(b) With 1.1 Moles of Phosphorus Oxychloride (18 Hours)—The product was a white powder which, after drying at 100°, had the following composition.

5.490 mg. substance: 0.568 cc. N_2 (751 mm. at 27°) (Dumas)
42.60 " " : 0.52 " " (760 " " 25°) (Van Slyke)
4.700 " " : 26.00 mg. ammonium phosphomolybdate
18.700 " " : 7.100 " $BaSO_4$
$C_{13}H_{16}O_7N_5PBa$. Calculated. N 13.40, amino N 2.68, P 5.94, Ba 26.29
Found. " 11.63, " " 0.68, " 8.03, " 22.34

The "excess" phosphorus, amounting to some 26 per cent of the total phosphorus present, was very readily hydrolyzed off by mineral acid as compared with that attached at position (5) of the sugar residue. The rest of the organic phosphorus was removed at the same rate as for authentic muscle adenylic acid.

Owing to the variability in composition of the product, it was decided to prepare the N-phospho 5-phospho derivative, 2 or 2.2 moles of phosphorus oxychloride being used during 18 hours. Under these conditions, the products obtained in a number of different experiments had similar compositions.

(*c*) *With 2.2 Moles of Phosphorus Oxychloride* (*18 Hours*)—On treating 3.0 gm. of dry, recrystallized monoacetone adenosine with 2.2 cc. of phosphorus oxychloride in dry pyridine and proceeding as usual,[1] the first precipitate was a white powder (5.3 gm.) which, after drying at 100°, had the following composition.

5.675 mg. substance: 0.412 cc. N_2 (758 mm. at 27.5°)
5.200 " " : 34.180 mg. ammonium phosphomolybdate
46.32 " " : 27.610 " $BaSO_4$
$C_{13}H_{15}O_{10}N_5P_2Ba_2$. Calculated. N 9.49, P 8.41, Ba 37.23
Found. " 8.22, " 9.54, " 35.07

Presumably owing to the excess of phosphorylating agent employed, the product contained some inorganic barium phosphate which accounts for the high phosphorus and low nitrogen figures. By colorimetric determination, the inorganic phosphorus was found to amount to 10 per cent of the total phosphorus present.

The mother liquor from the first precipitate was evaporated to dryness, and the material dissolved in water and precipitated with absolute ethanol, giving a second portion (2.5 gm.) having the following composition.

8.725 mg. substance: 0.656 cc. N_2 (776 mm. at 23°)
3.780 " " : 25.703 mg. ammonium phosphomolybdate
33.410 " " : 19.485 " $BaSO_4$
$C_{13}H_{15}O_{10}N_5P_2Ba_2$. Calculated. N 9.49, P 8.41, Ba 37.23
Found. " 8.85, " 9.87, " 34.32

Acid Hydrolysis of Phosphorylation Product from Monoacetone Adenosine—A sample of crude barium salt of diphospho acetone adenosine having the following composition was used for a typical experiment.

Found. N 9.19, P 10.20, Ba 29.13

By treatment with N hydrochloric acid during 7 minutes at 100°, 45 per cent of the total phosphorus was liberated as inorganic phosphate.

The same result was achieved at room temperature (but *without* appreciable simultaneous hydrolysis of the purine residue) after the material had stood for some 3 days, as indicated by the following experiment. To 0.1063 gm. dissolved in 5 cc. of N hydrochloric acid, 0.58 cc. of 2 N hydrochloric acid was added, and the solution rapidly diluted to 10 cc. with N hydrochloric acid. The solution was kept at room temperature and the free inorganic phosphorus liberated (in per cent of the total organic phosphorus) was estimated colorimetrically after the elapse of various intervals of time, with the following results: 3.7 (45 minutes), 26.9 (20.25 hours), and 43.0 per cent (68.25 hours).

A specimen of the still slightly impure barium 5-phosphoadenosine, prepared in this manner on a larger scale, was dissolved in water and the barium quantitatively removed by the addition of dilute sulfuric acid. After the barium sulfate was centrifuged off and the solution evaporated to small volume under diminished pressure, neutral lead acetate was added until no more precipitate formed. The lead precipitate was well washed, suspended in water, and decomposed with hydrogen sulfide. The filtrate was evaporated to 10 cc. under diminished pressure and acetone was added until no more precipitate formed.

The product was a white, amorphous substance which was not quite pure enough to be obtained crystalline, but which had a composition approximately that of adenylic acid.

4.412 mg. substance: 5.700 mg. CO_2 and 1.610 mg. H_2O
5.820 " " : 0.990 cc. N_2 (763.5 mm. at 24°)
4.878 " " : 27.410 mg. ammonium phosphomolybdate
$C_{10}H_{14}O_7N_5P$. Calculated. C 34.56, H 4.1, N 20.18, P 8.94
Found. " 35.23, " 4.1, " 19.38, " 8.16

Phosphorylation of Diacetyl Adenosine—Diacetyl adenosine was phosphorylated with 1.1 moles of phosphorus oxychloride in order to determine whether there would be preferential esterification at position (5) rather than on the amino group. The two groups were found to be of approximately equal reactivity, so that a product having a composition corresponding to a *mono-*

phospho derivative was obtained, but about half the organic phosphorus was labile (in the presence of N hydrochloric acid during 7 minutes at 100°).

To a solution of 0.84 cc. of phosphorus oxychloride in 10 cc. of dry pyridine cooled to −40° was added a solution of 3 gm. of dry, recrystallized diacetyl adenosine in 50 cc. of dry pyridine cooled to −30°. The mixture was cooled to −35° and then allowed to stand in an ice-salt bath at −10° to −15° during 2 hours. The clear, colorless solution was now recooled to −35°, whereupon some white material separated. 10 cc. of 90 per cent aqueous pyridine were added dropwise, with vigorous stirring and cooling to prevent the temperature from rising above −20°. During this treatment the white precipitate redissolved.

Filtered ice water (20 cc.) was now added (a white material being precipitated), followed by cold, saturated barium hydroxide solution until the mixture was faintly alkaline to thymolphthalein.

On standing, the mixture became less alkaline (owing to hydrolysis of the acetyl groups) and more barium hydroxide solution was added dropwise until alkalinity was maintained for 20 minutes. The mixture was evaporated to dryness under diminished pressure, more white precipitate forming as the evaporation proceeded. The dry product was shaken with 50 cc. of water and the mixture centrifuged. The precipitate was shaken with 100 cc. of water, centrifuged, and then dried by washing successively with 50 per cent ethyl alcohol, absolute ethanol, acetone, and dry ether. It was dried in a vacuum desiccator. Weight, 2.5 gm.

This product contained organic phosphate and gave a strong test for pentose with orcinol. The white, powdery material was dried at 100° and 15 mm. over phosphorus pentoxide during 24 hours and then had the following composition.

4.500 mg. substance: 0.550 cc. N_2 (764 mm. at 29°)
4.500 " " : 22.600 mg. ammonium phosphomolybdate
$C_{10}H_{12}O_7N_5PBa$. Calculated, N 14.52, P 6.43; found, N 13.88, P 7.29

Of the total phosphorus contained in this first precipitate, some 11 per cent was inorganic (possibly owing to the excess of phosphorus oxychloride employed) and 48 per cent was labile.

Acid Hydrolysis of Phosphorylation Product from Diacetyl Adenosine—2.5 gm. of the phosphorylation product from diacetyl adenosine were dissolved in 100 cc. of N hydrochloric acid and the solution kept at room temperature during 3 days. Cold, saturated barium hydroxide solution was now added until the mixture was alkaline to thymolphthalein. The precipitate was centrifuged off, washed twice by centrifuging with water, and dried in the usual manner. It consisted essentially of barium phosphate but, as in the parallel experiment with diphospho acetone adenosine, contained some unhydrolyzed starting material and some adsorbed end-product. It had a nitrogen content of 7.41 per cent.

The solution and washings from the precipitate were united and a few drops of acetic acid were added until the solution was just not alkaline to phenolphthalein. The solution was evaporated to dryness and the product dissolved in 114 cc. of water. Addition of 106 cc. of absolute ethanol gave a white flocculent precipitate which was centrifuged off and dried in the usual way. The white, powdery material (weight, 0.5 gm.) had the following composition, agreeing fairly well with that for barium adenylate.

6.502 mg. substance: 0.833 cc. N_2 (753 mm. at 28°)
3.478 " " : 14.524 mg. ammonium phosphomolybdate
26.332 " " : 10.00 " $BaSO_4$

$C_{10}H_{12}O_7N_5PBa$. Calculated. N 14.52, P 6.43, Ba 28.47
Found. " 14.40, " 6.18, " 22.34

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ROTATORY DISPERSION OF CONFIGURATIONALLY RELATED AMINES

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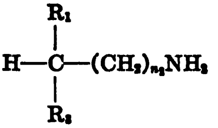
The present communication deals with three points regarding the configurational relationships of aliphatic amines of the general type



$$H-\underset{R_2}{\overset{R_1}{C}}-(CH_2)_{n_2}NH_2$$

The first is concerned with the direction of rotation in correlated secondary amines ($n_2 = 0$) and primary amines ($n_2 > 0$); the second with the direction of rotation in the members of the series of secondary amines homologous with respect to R_1; the third with the comparison of the dispersion curves of the amines and those of the corresponding alcohols.

The correlation of the configurations of primary and secondary amines can be arrived at by arguments identical with those employed in the case of alcohols;[1] namely, by comparing the direction of rotation in the accompanying four amines (I to IV).

In the three amines (I), (III), and (IV), the substituents are arranged counter-clockwise;[2] in (II) the order is reversed. Inasmuch as it is generally accepted that in hydrocarbons, alcohols, and amines the direction of rotation is determined by the clock-

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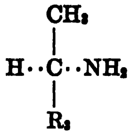
[1] Levene, P. A., and Rothen, A., *J. Biol. Chem.*, 116, 209 (1936).

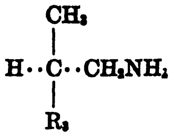
[2] The abbreviated expression "clockwise or counter-clockwise" is used in the following sense. When a molecule is viewed with the largest group toward the observer, the three other groups are arranged in clockwise or counter-clockwise order according to diminishing volume.



$$\begin{array}{c}\mathrm{CH_3}\\ |\\ \mathrm{H\cdot\cdot C\cdot\cdot NH_2}\\ |\\ \mathrm{R_3}\end{array}$$

Dextro

I

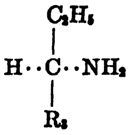


$$\begin{array}{c}\mathrm{CH_3}\\ |\\ \mathrm{H\cdot\cdot C\cdot\cdot CH_2NH_2}\\ |\\ \mathrm{R_3}\end{array}$$

$(R_3 > C_2H_5 > NH_2)$

Levo

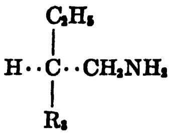
II



$$\begin{array}{c}\mathrm{C_2H_5}\\ |\\ \mathrm{H\cdot\cdot C\cdot\cdot NH_2}\\ |\\ \mathrm{R_3}\end{array}$$

Dextro

III



$$\begin{array}{c}\mathrm{C_2H_5}\\ |\\ \mathrm{H\cdot\cdot C\cdot\cdot CH_2NH_2}\\ |\\ \mathrm{R_3}\end{array}$$

Dextro

IV

wise or counter-clockwise arrangement of the substituents, it follows that substances (I), (III), and (IV) should be of one sign and substance (II) of the other.

In order to justify this conclusion it was necessary to show that the rotatory dispersions of substances (II) and (IV) are both normal. From Table V it can be seen that this is the case.

The direction of rotation in members of the series of secondary amines homologous with respect to R_1 was unsettled, since it was observed that the members of the corresponding series of azides, from which the amines were derived by reduction, differed in sign in members having $R_1 = CH_3$ or C_2H_5 and in those having $R_1 > C_2H_5$, even though all the azides were obtained from iodides having the same sign of rotation and similar rotatory dispersion.

Analysis of the dispersion curves of the amines, however, did not give a clear cut answer, as will be seen from the discussion in the experimental part. The difficulty arose from the fact that the nearest to the visible absorption regions of the amines are not anisotropic. It was therefore decided to study such derivatives of the amines as would have absorption bands nearer to the visible region. The benzoyl derivatives were chosen for this purpose. Analysis of the latter indicates that in the series of secondary amines homologous with respect to R_1 all members rotate in the same direction.

Rotatory Dispersion of Primary Amines—It was found that the direction of rotation of primary amines is of the same sign as that of the corresponding alcohols. It has been found that corresponding rotatory dispersions likewise are similar. As in the alcohols, so in the amines, the absorption bands nearest to the visible region are not anisotropic.

Inasmuch as the configurational relationships between secondary and primary amines are similar to those of primary and secondary alcohols, it may be justified to conclude that amines, as a rule, rotate in the same direction as the corresponding alcohols.

SUMMARY

1. The correlation of the configuration of primary and secondary amines is similar to that of the primary and secondary alcohols.

2. The direction of rotation of primary and of secondary amines is identical with that of corresponding alcohols.

3. In all alkyl amines the absorption regions nearest to the visible region are not anisotropic.

EXPERIMENTAL

Absorption Spectra—The absorption curves of many simple secondary and primary amines have recently been determined.[3] For all of them the absorption starts from $\simeq \lambda$ 2400 and increases very rapidly for shorter wave-lengths ($\epsilon_{2300} \simeq 50$ in hexane solution). The absorption curves of the hydrochlorides are displaced towards higher frequencies by about 200 Å.

Rotatory Dispersion Curves—Rotatory dispersion data are summarized in Tables II to V. It appears that the first absorption region, λ 2400 to λ 2000, is not anisotropic. This is in accord with what was found for so many other substances. Contrary to the rule of Kuhn, the first absorption region of substances with one chromophoric group is as a rule inactive, aliphatic aldehydes being an exception.

Secondary Amines—It may be seen from Table I that all secondary free amines homologous with respect to R_1 have the same sign of rotation. Identity of direction of rotation in members of homologous series generally implies identity of sign of corresponding partial rotations, since in numerous cases it was found that *the partial rotation of a band of the functional group has the*

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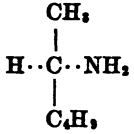
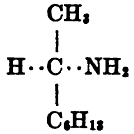
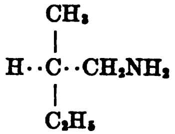
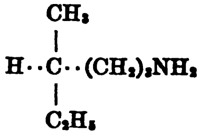
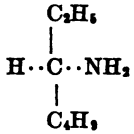
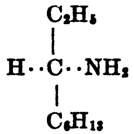
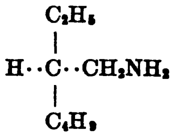
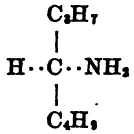
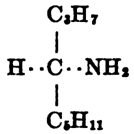
[3] Grunfeld, M., *Ann. chim.*, series 10, **20**, 304 (1933).

TABLE I

Configurationally Related Amines $[M]^{25}_{5780}$ *(Approximate Maximum Values)*

 $H\cdot\cdot C(CH_3)(C_4H_9)\cdot\cdot NH_2$ Free amine +4.8° HCl −2.9° Benzoyl +31.4°	 $H\cdot\cdot C(CH_3)(C_6H_{13})\cdot\cdot NH_2$ Free amine +6.8° HCl −10.2°	 $H\cdot\cdot C(CH_3)(C_2H_5)\cdot\cdot CH_2NH_2$ Free amine −5.2°	 $H\cdot\cdot C(CH_3)(C_2H_5)\cdot\cdot (CH_2)_2NH_2$ Free amine +12.4° HCl +13.9°
 $H\cdot\cdot C(C_2H_5)(C_4H_9)\cdot\cdot NH_2$ Free amine +4.2° HCl +1.7° Benzoyl +6.1°	 $H\cdot\cdot C(C_2H_5)(C_6H_{13})\cdot\cdot NH_2$ Free amine +7.2° HCl + or − Benzoyl +13.9°	 $H\cdot\cdot C(C_2H_5)(C_4H_9)\cdot\cdot CH_2NH_2$ Free amine +3.2° HCl +8.4°	
 $H\cdot\cdot C(C_3H_7)(C_4H_9)\cdot\cdot NH_2$ Free amine +0.6° HCl −1.9° Benzoyl +2.2°	 $H\cdot\cdot C(C_3H_7)(C_5H_{11})\cdot\cdot NH_2$ Free amine +0.7° HCl −1.5°		

same sign in all members of homologous series (it being understood that for all members considered $R_1 > R_3$ or $R_3 > R_1$).

The results of the analysis of the rotatory dispersion curves of the secondary free amines homologous with respect to R_1 seem to contradict that rule (Tables II and III). Thus, the dispersion constant of 2-amino-octane is low ($\lambda_0^2 = 0.0185$ for a one Drude term formula) and measurements in the ultraviolet region indicate that the dispersion is anomalous. On the other hand, the dispersion constant of 4-aminooctane or 4-aminononane is high ($\lambda_0^2 = 0.042$ or 0.050 respectively), indicating that the dispersion is normal and should be expressed by two terms of opposite sign. It may be that the system of optically active absorption bands in amines is of such complexity that closely located absorption bands anisotropic when $R_1 = CH_3$ and inactive when $R_1 = C_3H_7$, or *vice versa*, are the cause of these anomalies. It is perhaps significant that the value of λ_0^2 increases progressively in members of the series homologous with respect to R_1.

The difficulty encountered in the interpretation of the dispersion of the free amines is met also in the case of the hydrochlorides, as appears from Tables II and III. All hydrochlorides are opposite in sign to the free amines with the exception of 3-aminoheptane. It should be noticed that hydrochlorides of the amines having $R_1 = CH_3$ are more dispersive than the free amines and *vice versa* for the substances having $R_3 = C_3H_7$. As seen from Table III, the rotation of the hydrochloride of 3-aminononane can be of either sign, depending upon concentration. This offers no difficulty since in one case the dispersion is anomalous and normal in the other.

No difficulty of interpretation is encountered in the benzoyl derivatives (see Table IV). All of them have the same sign of rotation and their rotatory dispersion is normal.

The dispersion constant of the benzoyl derivative of 4-aminononane is especially high, undoubtedly because the absolute magnitudes of the first two opposite contributions are closer than in the other benzoyl derivatives; hence the low values for the molecular rotation.

Primary Amines—The results in the primary amines parallel entirely those observed in the corresponding alcohols. As can be seen from Table V, the amine with $n_2 = 1$ has a high dispersive

TABLE II

Rotatory Dispersion of Configurationally Related Secondary Amines in Homogeneous State and Their Hydrochlorides in Aqueous Solution

λ	Dextro-2-aminohexane d_4^{25} = 0.755 (*in vacuo*)				Dextro-2-aminooctane d_4^{25} = 0.771; n_D^{25} = 1.4220				Dextro-3-aminoheptane d_4^{25} = 0.7628 (*in vacuo*); n_D^{25} = 1.4163			
	Free amine l = 5 cm. $[M]^{25}_{max.} = \frac{1.514}{\lambda^2 - 0.019}$		Hydrochloride c = 1.66 M; l = 20 cm. $[M]^{25}_{max.} = -\frac{0.889}{\lambda^2 - 0.024}$		Free amine l = 10 cm.; ultraviolet l = 5 cm. $[M]^{25}_{max.} = \frac{2.155}{\lambda^2 - 0.0185}$		Hydrochloride c = 0.881 M; l = 20 cm.; ultraviolet l = 5 cm. $[M]^{25}_{max.} = -\frac{3.170}{\lambda^2 - 0.0248}$		Free amine l = 20 cm.; ultraviolet l = 5 cm. $[M]^{25}_{max.} = \frac{1.279}{\lambda^2 - 0.028}$		Hydrochloride c = 1.61 M; l = 40 cm.; ultraviolet l = 10 cm. $[M]^{25}_{max.} = \frac{5.282}{\lambda^2 - 0.032}$	
	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$	α^{25}	$[M]^{25}_{max.}$
5892.6					3.233	6.58	−1.434	−9.89				
5875.6	1.605	4.67	−0.861	−2.82					3.287	4.020	0.676	1.702
5780.1	1.651	4.80	−0.877	−2.87	3.355	6.828	−1.487	−10.25	3.417	4.179	0.695	1.750
5460.7	1.857	5.40	−0.989	−3.24	3.797	7.728	−1.689	−11.65	3.872	4.735	0.790	1.989
4358.3	3.04	8.85	−1.64	−5.36	6.19_5	12.61	−2.79	−19.24	6.46	7.90	1.33	3.35
4046.6	3.47	10.1			7.29	14.84	−3.31	22.82	7.69	9.40	1.57	3.95
3920					3.90	15.9						
3560									2.60	12.7		
3340									3.10	15.2		
3250					−5.90	24.0			3.60	17.6		
3180												
3100									3.85	18.8		
3060							−1.65	−45.5				
3040					−6.90	28.1						
3020									4.10	20.0		

3000											0.9	9.1
2970									4.35	21.3		
2920									4.60	22.5		
2880											1.04	10.0
2830							−2.15	−59.3				
2750					−0.87 (0.5 cm.)	35.4						
2730							−2.40	−66.2				
2650							−2.65	−73.1				
2500					−0.87 (0.5 cm.)	35.4						
2470							−3.15	−86.9				
2370							−3.65	−101				

TABLE III

Rotatory Dispersion of Configurationally Related Secondary Amines in Homogeneous State and Their Hydrochlorides in Aqueous Solution

λ	Dextro-3-aminononane						Dextro-4-aminooctane $d_4^{25} = 0.7702$ (*in vacuo*)				Dextro-4-aminononane $d_4^{25} = 0.7772$ (*in vacuo*)			
	Free amine		Hydrochloride				Free amine		Hydrochloride		Free amine		Hydrochloride	
	l = 10 cm.		c = 1.134 M; l = 20 cm.		c = 0.283 M; l = 40 cm.		l = 20 cm.		c = 2.17 M; l = 20 cm.		l = 10 cm.		c = 1.05 M; l = 40 cm.	
	$[M]^{25}_{\text{max.}} = \frac{2.200}{\lambda^2 - 0.027}$		$[M]^{25}_{\text{max.}} = -\frac{0.285}{\lambda^2}$		$[M]^{25}_{\text{max.}} = \frac{1.327}{\lambda^2 - 0.037}$		$[M]^{25}_{\text{max.}} = \frac{0.172}{\lambda^2 - 0.0424}$		$[M]^{25}_{\text{max.}} = -\frac{0.579}{\lambda^2 - 0.029}$		$[M]^{25} = \frac{0.187}{\lambda^2 - 0.050}$		$[M]^{25} = -\frac{0.460}{\lambda^2 - 0.026}$	
	α^{25}	$[M]^{25}_{\text{max.}}$	α^{25}	$[M]^{25}_{\text{max.}}$	α^{25}	$[M]^{25}_{\text{max.}}$	α^{25}	$[M]^{25}_{\text{max.}}$	α^{25}	$[M]^{25}_{\text{max.}}$	α^{25}	$[M]^{25}$	^{25}	$[M]^{25}$
5875.6	3.571	6.963			0.473	4.36	0.340	0.570					−0.605	−1.44
5780.1	3.694	7.203	−0.185	−0.853	0.484	4.46	0.351	0.588	−0.822	−1.895	0.358	0.659	−0.629	−1.50
5460.7	4.182	8.155	−0.207	−0.954	0.550	5.07	0.401	0.672	−0.920	−2.122	0.405	0.746	−0.709	−1.69
4358.3	6.97	13.59	−0.325	−1.50	0.94	8.66	0.695	1.16	−1.56	−3.60	0.73	1.34	−1.18	−2.81
4046.6	8.31	16.20	−0.37	−1.70	1.13	10.4	0.835	1.40	−1.85	−4.26	0.93	1.71	−1.39	−3.32

power, the dispersion constant of a single Drude term $\lambda_0^2 = 0.0585$ being too high to correspond to the position of the first active absorption band. Consequently the rotatory power is the sum of two terms of opposite sign whose magnitudes are of the same order. This is in harmony with the low absolute value of the molecular rotation.

The amine with $n_2 = 3$ is less dispersive and the value of the dispersion constant $\lambda_0^2 = 0.022$ is about the same as that found for the corresponding carbinol.

A similarity in the value of the molecular rotations corresponds to this similarity in the dispersive power of amines and alcohols. It thus shows that the analogy in the system of optically active absorption bands must be in fact very close in both types of compounds. For instance, for 2-methyl-1-aminobutane, $[M]_{5876}^{25} = -5.1°$, and for the corresponding alcohol, 2-methylbutanol-1, $[M]_{5876}^{25} = -5.2°$; for 4-methyl-1-aminohexane, $[M]_{5876}^{25} = 12.0°$, and for the corresponding alcohol, 4-methylhexanol-1, $[M]_{5876}^{25} = 11.9°$.

TABLE IV

Rotatory Dispersion of Benzoyl Derivatives of Configurationally Related Secondary Amines

λ	Dextro-benzoyl of dextro-2-aminohexane in alcohol		Dextro-benzoyl of dextro-3-aminononane in alcohol		Dextro-benzoyl of dextro-4-aminooctane in alcohol	
	$c = 0.421$ M; $l = 20$ cm. $[M]_{\text{max.}}^{25} = \frac{9.606}{\lambda^2 - 0.0404}$		$c = 0.532$ M; $l = 20$ cm. $[M]_{\text{max.}}^{25} = \frac{4.198}{\lambda^2 - 0.033}$		$c = 0.776$ M; $l = 20$ cm. $[M]_{\text{max.}}^{25} = \frac{0.567}{\lambda^2 - 0.076}$	
	α^{25}	$[M]_{\text{max.}}^{25}$	α^{25}	$[M]_{\text{max.}}^{25}$	α^{25}	$[M]_{\text{max.}}^{25}$
5875.6	2.474	31.44				
5780.1	2.572	32.69	1.425	13.93	0.335	2.16
5460.7	2.934	37.29	1.620	15.83	0.395	2.54
4358.3	5.05	64.2	2.73	26.70	0.75	4.83
4046.6	6.13	77.8				

Preparation

Dextro-Benzoyl-2-Aminobutane—2 gm. of 2-aminobutane hydrochloride, $[M]_D^{25} = -0.44°$ (in water),[4] were converted into the benzoyl derivative by the method of Schotten and Baumann. It

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[4] Levene, P. A., and Rothen, A., *J. Biol. Chem.*, **115**, 415 (1936).

TABLE V

Rotatory Dispersion of Configurationally Related Primary Amines in Homogeneous State and Their Corresponding Hydrochlorides in Aqueous Solution

λ	Levo-2-methyl-1-aminobutane d_4^{25} = 0.750 (*in vacuo*)		Dextro-4-methyl-1-aminohexane d_4^{25} = 0.7709 (*in vacuo*); n_D^{25} = 1.4220				Dextro-2-ethyl-1-aminohexane d_4^{25} = 0.7844 (*in vacuo*); n_D^{25} = 1.4286			
	Free amine l = 5 cm. $[M]_{max.}^{25} = -\frac{1.441}{\lambda^2 - 0.0585}$		Free amine l = 20 cm.; ultraviolet l = 5 cm. $[M]_{max.}^{25} = \frac{3.876}{\lambda^2 - 0.0222}$		Hydrochloride c = 1.412 M; l = 40 cm. $[M]_{max.}^{25} = \frac{4.335}{\lambda^2 - 0.0232}$		Free amine l = 20 cm. $[M]_{max.}^{25} = \frac{0.974}{\lambda^2 - 0.028}$		Hydrochloride c = 1.983 M; l = 40 cm. $[M]_{max.} = \frac{2.506}{\lambda^2 - 0.0381}$	
	α^{25}	$[M]_{max.}^{25}$	α^{25}	$[M]_{max.}^{25}$	α^{25}	$[M]_{max.}^{25}$	α^{25}	$[M]_{max.}^{25}$	α^{25}	$[M]_{max.}^{25}$
5875.6	−0.923	−5.10	5.755	12.00	2.744 (?)	13.58	0.808	3.05	1.405	8.15
5780.1	−0.953	−5.26	5.957	12.42	2.813	13.92	0.845	3.19	1.460	8.47
5460.7	−1.087	−6.00	6.735	14.04	3.184	15.76	0.955	3.60	1.660	9.63
4358.3	−1.99	−11.0	11.08	23.10	5.25	25.98	1.595	6.02	2.84	16.50
4046.6	−2.42	−13.4	13.13	27.38	6.23	30.83	1.90	7.17	3.44	19.95
3740			3.90	32.5						
3590			4.40	36.7						
3420			4.90	40.9						
3300			5.40	45.0						
3170			5.90	49.2						
3080			6.40	53.4						
2995			6.90	57.5						
2835			7.90	65.9						
2760			8.40	70.0						
2530			1.0 (0.5 cm.)	83.4						
2490			1.1 (0.5 ")	91.7						

was recrystallized twice by dissolving it in ether and then adding pentane until crystals appeared. M.p. 86–88°.

$$[\alpha]^{25}_{D} = \frac{+0.40° \times 100}{1 \times 6.0} = +6.7°; \quad [M]^{25}_{D} = +11.8° \text{ (in absolute alcohol)}$$

4.706 mg. substance: 12.915 mg. CO_2 and 3.580 mg. H_2O

$C_{11}H_{15}ON$.	Calculated.	C 74.52, H 8.54
177.1	Found.	" 74.83, " 8.51

Dextro-2-Aminooctane—0.8 gm. of 2-aminooctane hydrochloride

$$[\alpha]^{25}_{D} = \frac{-0.55° \times 100}{1 \times 10.0} = -5.5° \text{ (in water)}^{4}$$

were converted into the benzoyl derivative. M.p. 73–74°.

$$[\alpha]^{25}_{D} = \frac{+2.85° \times 100}{1 \times 10.0} = +28.5°; \quad [M]^{25}_{D} = +66.2° \text{ (in absolute alcohol)}$$

3.376 mg. substance: 9.547 mg. CO_2 and 3.005 mg. H_2O

$C_{15}H_{22}ON$.	Calculated.	C 77.52, H 9.55
232.2	Found.	" 77.11, " 9.96

Dextro-2-Hexanol—2-Hexanol was prepared from *n*-butyl magnesium bromide and acetaldehyde. The carbinol was resolved as described by Pickard and Kenyon.[5] The insoluble strychnine salt gave a carbinol which boiled at 99–100°, p = 168 mm.

$$[\alpha]^{25}_{D} = \frac{+8.72°}{1 \times 0.813} = +10.7°; \quad [M]^{25}_{D} = +10.9° \text{ (homogeneous)}$$

3.304 mg. substance: 8.535 mg. CO_2 and 4.080 mg. H_2O

$C_6H_{14}O$.	Calculated.	C 70.51, H 13.82
102.1	Found.	" 70.44, " 13.81

Levo-2-Iodohexane—20 gm. of 2-hexanol, $[\alpha]^{25}_{D} = +10.7°$ (homogeneous), were treated with anhydrous hydrogen iodide as described.[4] The sealed bomb tube was allowed to stand at room temperature overnight. The iodide boiled at 90–91°, $p = 70$ mm. Yield 38 gm.; $n_D^{25} = 1.4911$.

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[5] Pickard, J., and Kenyon, R. H., *J. Chem. Soc.*, **99**, 58 (1911).

$$[\alpha]_D^{25} = \frac{-43.6^\circ}{1 \times 1.42} = -30.7^\circ; \qquad [M]_D^{25} = -65.1^\circ \text{ (homogeneous)}$$

3.610 mg. substance: 4.490 mg. CO_2 and 2.005 mg. H_2O
$C_6H_{13}I$. Calculated. C 33.96, H 6.18
212.0 Found. " 33.89, " 6.21

Dextro-2-Azidohexane—38 gm. of 2-iodohexane, $[\alpha]_D^{25} = -30.7^\circ$ (homogeneous), were added to a solution of 14 gm. of sodium azide (Kahlbaum) in 38 cc. of water and 400 cc. of methanol. This was sealed into four bomb tubes and heated at 80° for 16 hours. The azide was isolated as usual.[4] B.p. 96–98°, $p = 160$ mm. Yield 17 gm.; $d_4^{25} = 0.8571$ (*in vacuo*); $n_D^{25} = 1.4253$.

$$[\alpha]_D^{25} = \frac{+23.85^\circ}{1 \times 0.857} = +27.8^\circ; \qquad [M]_D^{25} = +35.3^\circ \text{ (homogeneous)}$$

3.515 mg. substance: 7.320 mg. CO_2 and 3.310 mg. H_2O
$C_6H_{13}N_3$. Calculated. C 56.64, H 10.31
127.1 Found. " 56.79, " 10.53

Levo-2-Aminohexane—17 gm. of 2-azidohexane, $[\alpha]_D^{25} = +27.8^\circ$ (homogeneous), were dissolved in 35 cc. of methanol and 0.7 gm. of Adams' catalyst was added. This was shaken with hydrogen at a pressure of 3 atmospheres for 5 hours. The amine hydrochloride was isolated as usual.[6] Yield 7.5 gm.

$$[\alpha]_D^{25} = \frac{-2.27^\circ \times 100}{2 \times 20.0} = -5.68^\circ; \qquad [M]_D^{25} = -7.82^\circ \text{ (absolute alcohol)}$$

4.151 mg. substance: 8.000 mg. CO_2 and 4.380 mg. H_2O
$C_6H_{16}NCl$. Calculated. C 52.33, H 11.72
137.6 Found. " 52.55, " 11.80

The salt was converted into the free base as usual. B.p. 70°, $p = 155$ mm. $d_4^{27} = 0.755$ (*in vacuo*).

$$[\alpha]_D^{27} = \frac{+3.25^\circ}{1 \times 0.755} = +4.30^\circ; \qquad [M]_D^{27} = +4.35^\circ \text{ (homogeneous)}$$

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[6] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **115**, 267 (1936).

Dextro-Benzoyl-2-Aminohexane—1.5 gm. of 2-aminohexane hydrochloride, $[\alpha]^{25}_{D} = -5.68°$ (absolute alcohol), were converted into the benzoyl derivative. M.p. 86–88°.

$$[\alpha]^{25}_{D} = \frac{+2.85° \times 100}{1 \times 20.0} = +14.3°; \qquad [M]^{25}_{D} = +29.3° \text{ (in absolute alcohol)}$$

3.982 mg. substance: 11.080 mg. CO_2 and 3.325 mg. H_2O
$C_{13}H_{19}ON$. Calculated. C 76.04, H 9.33
205.2 Found. " 75.89, " 9.34

Dextro-3-Heptanol—The inactive carbinol was prepared from *n*-butyl magnesium bromide and propionaldehyde as usual. It was resolved according to the procedure of Pickard and Kenyon.[7] The less soluble salt gave a carbinol which boiled at 104–106°, p = 117 mm.

$$[\alpha]^{25}_{D} = \frac{+4.20°}{1 \times 0.820} = +5.12°; \qquad [M]^{25}_{D} = +5.94° \text{ (homogeneous)}$$

4.320 mg. substance: 11.483 mg. CO_2 and 5.375 mg. H_2O
$C_7H_{16}O$. Calculated. C 72.33, H 13.89
116.1 Found. " 72.48, " 13.92

Levo-3-Iodoheptane—60 gm. of 3-heptanol, $[\alpha]^{25}_{D} = +5.12°$ (homogeneous), were treated with anhydrous hydrogen iodide as usual. The three sealed bomb tubes were allowed to stand overnight at room temperature. The iodide distilled at 76°, p = 12 mm. Yield 78 gm.; $d^{25}_{4} = 1.3675$ (*in vacuo*); $n^{25}_{D} = 1.4901$.

$$[\alpha]^{25}_{D} = \frac{-11.3°}{1 \times 1.37} = -8.25°; \qquad [M]^{25}_{D} = -18.6° \text{ (homogeneous)}$$

4.916 mg. substance: 6.695 mg. CO_2 and 2.905 mg. H_2O
$C_7H_{15}I$. Calculated. C 37.16, H 6.69
226.0 Found. " 37.13, " 6.61

Dextro-3-Azidoheptane—78 gm. of 3-iodoheptane, $[\alpha]^{25}_{D} = -8.25°$ (homogeneous), were added to a solution of 30 gm. of sodium azide (Kahlbaum) in 70 cc. of water and 750 cc. of methanol. The solution was sealed into nine bomb tubes and heated for 14 hours at 75°. The azide was isolated as usual. B.p. 79–81°, $p = 43$ mm. Yield 13 gm.; $d_4^{25} = 0.8583$ (*in vacuo*); $n_D^{25} = 1.4298$.

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[7] Pickard, J., and Kenyon, R. H., *J. Chem. Soc.*, **103**, 1944 (1913).

$$[\alpha]_D^{25} = \frac{+1.53°}{1 \times 0.858} = +1.78°; \quad [M]_D^{25} = +2.51° \text{ (homogeneous)}$$

3.115 mg. substance: 6.805 mg. CO_2 and 2.960 mg. H_2O
$C_7H_{15}N_3$. Calculated. C 59.52, H 10.71
141.1 Found. " 59.57, " 10.63

Dextro-3-Aminoheptane—13 gm. of 3-azidoheptane, $[\alpha]_D^{25} = +1.78°$ (homogeneous), were dissolved in 40 cc. of methanol, and 0.5 gm. of Adams' catalyst was added. This was shaken with hydrogen at a pressure of 3 atmospheres for 3 hours. The hydrochloride was isolated as usual.

$$[\alpha]_D^{25} = \frac{+0.18° \times 100}{1 \times 18.0} = +1.00°; \quad [M]_D^{25} = +1.52° \text{ (10\% HCl)}$$

The free base was isolated from the salt. B.p. 75°, $p = 70$ mm. $d_4^{25} = 0.783$ (*in vacuo*).

$$[\alpha]_D^{25} = \frac{+3.25°}{1 \times 0.783} = +4.15°; \quad [M]_D^{25} = +4.78° \text{ (homogeneous)}$$

$$[\alpha]_D^{25} = \frac{+0.26° \times 100}{1 \times 10.0} = +2.6°; \quad [M]_D^{25} = +3.0° \text{ (in absolute alcohol)}$$

2.985 mg. substance: 7.970 mg. CO_2 and 3.910 mg. H_2O
$C_7H_{17}N$. Calculated. C 72.95, H 14.88
115.1 Found. " 72.81, " 14.66

Dextro-Benzoyl-3-Aminoheptane—3-Aminoheptane hydrochloride, $[\alpha]_D^{25} = +1.00°$ (10 per cent HCl), was converted into its benzoyl derivative. M.p. 66–68°.

$$[\alpha]_D^{25} = \frac{+0.20° \times 100}{1 \times 10.0} = +2.0°; \quad [M]_D^{25} = +4.4° \text{ (in absolute alcohol)}$$

4.110 mg. substance: 11.510 mg. CO_2 and 3.398 mg. H_2O
$C_{14}H_{21}ON$. Calculated. C 76.65, H 9.66
219.2 Found. " 76.37, " 9.25

Dextro-3-Nonanol[8]—3-Nonanol was prepared from ethyl magnesium bromide and heptaldehyde. The carbinol was resolved by recrystallizing the cinchonidine salt of the phthalate from acetone. The less soluble salt gave a carbinol which boiled at 96–98°, $p = 19$ mm.

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[8] Pickard, J., and Kenyon, R. H., *J. Chem. Soc.*, 103, 1935 (1913).

$$[\alpha]_D^{25} = \frac{+5.83^\circ}{1 \times 0.823} = +7.08^\circ; \quad [M]_D^{25} = +10.2^\circ \text{ (homogeneous)}$$

3.498 mg. substance: 9.595 mg. CO_2 and 4.380 mg. H_2O

$C_9H_{20}O$. Calculated. C 74.92, H 13.98
144.2 Found. " 74.80, " 14.01

Levo-3-Iodononane—30 gm. of 3-nonanol, $[\alpha]_D^{25} = +7.08^\circ$ (homogeneous), were treated with anhydrous hydrogen iodide. The two sealed bomb tubes were allowed to stand overnight at room temperature. The iodide distilled at 99–100°, $p = 10$ mm. Yield 40 gm.

$$[\alpha]_D^{25} = \frac{-18.2^\circ}{1 \times 1.28} = -14.2^\circ; \quad [M]_D^{25} = -36.1^\circ \text{ (homogeneous)}$$

4.235 mg. substance: 6.600 mg. CO_2 and 2.905 mg. H_2O

$C_9H_{19}I$. Calculated. C 42.51, H 7.54
254.1 Found. " 42.52, " 7.67

Dextro-3-Azidononane—40 gm. of 3-iodononane, $[\alpha]_D^{25} = -14.2^\circ$ (homogeneous), were added to a solution of 12 gm. of sodium azide (Kahlbaum) in 40 cc. of water and 650 cc. of methanol. This was sealed into seven bomb tubes and heated for 10 hours at 100°. The azide was isolated as previously described. B.p. 105–107°, $p = 30$ mm. Yield 17 gm.; $d_4^{25} = 0.8575$ (*in vacuo*); $n_D^{25} = 1.4373$.

$$[\alpha]_{5780}^{25} = \frac{+5.22^\circ}{2 \times 0.858} = +3.04^\circ; \quad [M]_{5780}^{25} = +5.14^\circ \text{ (homogeneous)}$$

4.598 mg. substance: 10.800 mg. CO_2 and 4.600 mg. H_2O

$C_9H_{19}N_3$. Calculated. C 63.84, H 11.32
169.2 Found. " 64.05, " 11.19

Dextro-3-Aminononane—15 gm. of 3-azidononane, $[\alpha]_{5780}^{25} = +3.04^\circ$ (homogeneous), were dissolved in 40 cc. of methanol and 0.8 gm. of Adams' catalyst was added. This was shaken with hydrogen at a pressure of 3 atmospheres for 6 hours. The amine hydrochloride was isolated as usual.

$$[\alpha]_D^{25} = \frac{+0.09^\circ \times 100}{1 \times 6.0} = +1.5^\circ; \quad [M]_D^{25} = +2.7^\circ \text{ (in water)}$$

The free base was isolated and distilled from sodium. B.p. 102°, $p = 50$ mm. $d_4^{27} = 0.781$ (*in vacuo*).

$$[\alpha]_D^{27} = \frac{+3.60°}{1 \times 0.781} = +4.61°; \quad [M]_D^{27} = +6.60° \text{ (homogeneous)}$$

$$[\alpha]_D^{25} = \frac{+0.37° \times 100}{1 \times 10.0} = +3.7°; \quad [M]_D^{25} = +5.3° \text{ (in absolute alcohol)}$$

3.060 mg. substance: 8.475 mg. CO_2 and 4.010 mg. H_2O

$C_9H_{21}N$.	Calculated.	C 75.43, H 14.79
143.2	Found.	" 75.52, " 14.66

Dextro-Benzoyl-3-Aminononane—3-Aminononane hydrochloride, $[\alpha]_D^{25} = +1.50°$ (in water), was converted into the benzoyl derivative. M.p. 86°.

$$[\alpha]_D^{25} = \frac{+1.25° \times 100}{1 \times 10.0} = +12.5°; \quad [M]_D^{25} = +30.9° \text{ (in absolute alcohol)}$$

4.690 mg. substance: 13.422 mg. CO_2 and 4.200 mg. H_2O

$C_{16}H_{25}ON$.	Calculated.	C 77.67, H 10.19
247.2	Found.	" 78.04, " 10.00

Levo-4-Octanol[9]—4-Octanol was resolved by recrystallizing the strychnine salt of the phthalate from 90 per cent acetone. The less soluble portion gave a carbinol which boiled at 79–80°, $p =$ 17 mm.

$$[\alpha]_D^{25} = \frac{+0.52°}{1 \times 0.81} = +0.64°; \quad [M]_D^{25} = +0.83° \text{ (homogeneous)}$$

3.920 mg. substance: 10.61 mg. CO_2 and 4.860 mg. H_2O

$C_8H_{18}O$.	Calculated.	C 73.77, H 13.94
130.1	Found.	" 73.80, " 13.87

Levo-4-Iodooctane—20 gm. of 4-octanol, $[\alpha]_D^{25} = +0.64°$ (homogeneous), were treated with anhydrous hydrogen iodide as previously described. The iodide boiled at 97°, $p = 22$ mm. Yield 30 gm.; $d_4^{25} = 1.3233$ (*in vacuo*); $n_D^{25} = 1.4891$.

$$[\alpha]_D^{25} = \frac{-2.32°}{1 \times 1.32} = -1.76°; \quad [M]_D^{25} = -4.23° \text{ (homogeneous)}$$

5.158 mg. substance: 7.595 mg. CO_2 and 3.320 mg. H_2O

$C_8H_{17}I$.	Calculated.	C 39.99, H 7.14
240.1	Found.	" 40.15, " 7.20

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[9] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **91**, 405 (1931).

Levo-4-Azidooctane—30 gm. of 4-iodooctane, $[\alpha]_D^{25} = -1.76°$ (homogeneous), were added to a solution of 12 gm. of sodium azide (Kahlbaum) in 30 cc. of water and 400 cc. of methanol. This was sealed into four bomb tubes and heated for 16 hours at 80°. The azide was isolated as usual. B.p. 92–93°, $p = 35$ mm. Yield 15 gm.; $d_4^{25} = 0.8572$ (*in vacuo*); $n_D^{25} = 1.4342$.

$$[\alpha]_D^{25} = \frac{-0.70°}{1 \times 0.857} = -0.82°; \quad [M]_D^{25} = -1.27° \text{ (homogeneous)}$$

4.675 mg. substance: 10.630 mg. CO_2 and 4.600 mg. H_2O
$C_8H_{17}N_3$. Calculated. C 61.87, H 11.05
155.2 Found. " 62.06, " 11.01

Levo-4-Aminooctane—15 gm. of 4-azidooctane, $[\alpha]_D^{25} = -0.82°$ (homogeneous), were dissolved in 50 cc. of methanol and 1 gm. of Adams' catalyst was added. This was shaken with hydrogen at a pressure of 3 atmospheres for 6 hours. The hydrochloride was isolated as usual.

$$[\alpha]_D^{25} = \frac{-0.10° \times 100}{1 \times 20.0} = -0.50°; \quad [M]_D^{25} = -0.83° \text{ (10\% HCl)}$$

3.422 mg. substance: 7.255 mg. CO_2 and 3.730 mg. H_2O
$C_8H_{20}NCl$. Calculated. C 57.96, H 12.18
165.6 Found. " 57.81, " 12.19

The free base was liberated as usual. B.p. 92–93°, $p = 80$ mm. $d_4^{27} = 0.777$ (*in vacuo*).

$$[\alpha]_D^{27} = \frac{+0.35°}{1 \times 0.78} = +0.45°; \quad [M]_D^{27} = +0.58° \text{ (homogeneous)}$$

4.922 mg. substance: 13.410 mg. CO_2 and 6.440 mg. H_2O
$C_8H_{19}N$. Calculated. C 74.33, H 14.83
129.2 Found. " 74.29, " 14.64

Dextro-4-Aminooctane Benzoate—2 gm. of 4-aminooctane hydrochloride, $[\alpha]_D^{25} = -0.50°$ (10 per cent HCl), were converted into the benzoyl derivative. M.p. 99–100°.

$$[\alpha]_D^{25} = \frac{+0.26° \times 100}{1 \times 20.0} = +1.30°; \quad [M]_D^{25} = +3.03° \text{ (in absolute alcohol)}$$

3.570 mg. substance: 10.110 mg. CO_2 and 3.180 mg. H_2O
$C_{15}H_{23}ON$. Calculated. C 77.19, H 9.94
233.2 Found. " 77.22, " 9.97

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THE MECHANISM OF THE REACTION OF SUBSTITUTION AND WALDEN INVERSION

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It was emphasized in previous publications[1] that rigorous proof of the configurational relationship of secondary carbinols and corresponding halides and of hydroxy acids and corresponding halogeno acids had not yet been presented. On the other hand, it was established in our laboratory, on sufficient evidence, that amines and corresponding carbinols rotate in the same direction, and also that configurationally related secondary halides are of the same sign. Furthermore, Polanyi and Bergmann[2] as well as Hughes, Juliusburger, Masterman, Topley, and Weiss[3] reached the conclusion on good grounds that substitution of halogen for halogen is connected with an inversion of configuration.

It is now intended to use this information in an attempt to throw light on the configurational relationship of corresponding hydroxy and halogeno derivatives.

The argument on which this investigation is based is the following: If two consecutive reactions of substitution on the asymmetric carbon atom follow the same mechanism, either with or without inversion of configuration, then the final substance should have the configuration of the starting material.

In Table I are given a set of reactions through which it was attempted to obtain the desired information.

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[1] Levene, P. A., and Rothen, A., *J. Biol. Chem.*, **107**, 533 (1934). Levene, P. A., Rothen, A., and Marker, R. E., *J. Chem. Physic.*, **4**, 442 (1936).

[2] Bergmann, E., Polanyi, M., and Szabo, A. L., *Tr. Faraday Soc.*, **32**, 843 (1936). von Hartel, H., Meer, N., and Polanyi, M., *Z. physik. Chem., Abt. B*, **19**, 139 (1932).

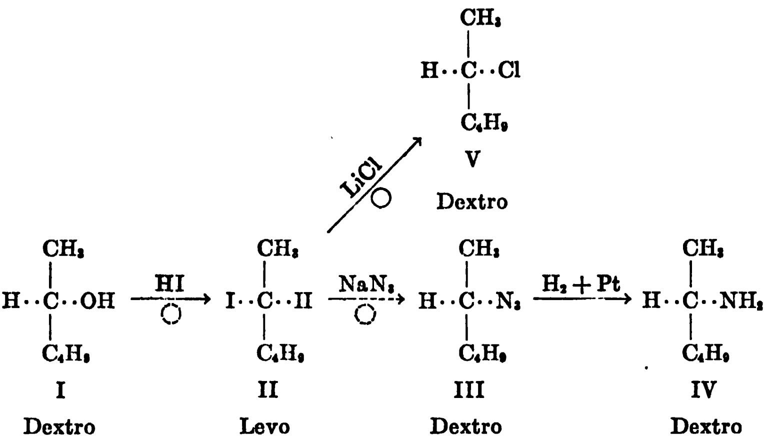
[3] Hughes, E. D., Juliusburger, F., Masterman, S., Topley, B., and Weiss, J., *J. Chem. Soc.*, 1525 (1935).

From Table I it can be seen that two consecutive reactions of substitution lead from a dextro-carbinol (I) to a dextro-amine (IV). It is evident that the amine (IV) is configurationally related to the azide (III); hence the azide (III) likewise is configurationally related to carbinol (I).

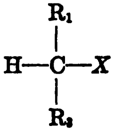
Unfortunately, there exists no definite information regarding the mechanism of either one of the two reactions of substitution. However, substitution of chlorine for iodine is connected with an inversion of configuration. True, doubt was expressed as to the identity of direction of rotation in two configurationally related secondary halides. This scepticism, however, is not warranted for the following reasons.

TABLE I

Reactions of Substitution in Saturated Derivatives



First, a series of substances of Type I



$$H-\overset{R_1}{\underset{R_2}{C}}-X$$

I

(where X = F, Cl, Br, I
R_1 and R_2 = normal alkyl groups)

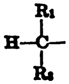
may in a sense be regarded as a homologous series, since the 4 halogen atoms are similar in their chemical properties and in the system of their absorption bands, but differ principally in their volume. Hence, if two members of this series differed in the direction of their rotation, the character of their rotatory dispersion should also be different. The latter, however, is not the case.

Second, as can be seen from Table II, levorotatory bromides and iodides all lead on the action of lithium chloride to dextrorotatory chlorides.

Hence, bromides and iodides rotating in the same direction are configurationally related. Thus it may be accepted that in the reactions given in Table I, substitution of the chlorine for the iodine atom is connected with an inversion of configuration. It may be mentioned that this is the first experimental evidence to the effect that substitution of a chlorine for another halogen is connected with an inversion of configuration.

TABLE II

Direction of Partial Rotations of Functional Groups —OH, —Br, —I, or —Cl

 $H-\overset{R_1}{\underset{R_2}{C}}-$		$-OH\rightarrow$	$-Br$ or $-I\xrightarrow{(LiCl)}$	$-Cl$
R_1	R_2			
n-Butyl	Vinyl	+	(Br) −	+
Ethyl	*n*-Hexyl	+	(I) −	+
n-Propyl	*n*-Butyl	+	" −	+
Ethyl	Phenyl	+	(Br) −	+
n-Propyl	"	+ or −	" −	+

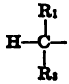
This reaction may serve as a key to explain the other reactions given in Table I. The azido group may be regarded as a pseudohalogen. It then seems legitimate to assume that the substitution of an azido group for a halogen atom proceeds similarly to that of 1 halogen atom for another. Therefore, the azido group, like a halogen atom, should approach the dipole from the positive end, and thus substitution should bring about an inversion of configuration. This assumption leads to the conclusion that the substitution of a halogen atom for the hydroxyl group in substances of Table I likewise is connected with an inversion of configuration. It may be seen from Table III that the set of reactions given in Table I has been observed in more than one case.

In their latest publication, Polanyi and Bergmann assumed that substitution of halogen for halogen is connected with inversion of configuration. It seems justified now to generalize this formulation for substances of Type I (X = a negative functional group) to the effect that substitution of any negative group or atom for another is connected with an inversion of configuration.

α-Substituted Carboxylic Acids—In α-substituted carboxylic acids a choice between the two possible mechanisms of the reaction of substitution is more difficult than in substances of Type I for the reason that ionization, lactone formation, enolization, etc., furnish frequently disturbing concomitant reactions; in addition, in the α-bromo-, or α-chlorocarboxylic acids, it is difficult to distinguish the partial rotations of the halogen atom from those of the carboxyl group. However, from the knowledge of configurational relationship of substances of Type I and of corresponding α-carboxylic acids, it is possible to correlate the mechanism of the reaction of substitution of these two groups of substances.

TABLE III

Direction of Rotations of Functional Groups OH, Hl, N_3, NH_2

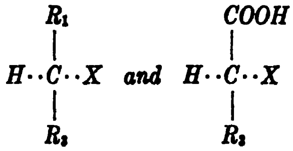
 $H-\overset{R_1}{\underset{R_2}{C}}-$		—OH →	—Hl →	$-N_3$ →	$-NH_2$
R_1	R_2				
n-Butyl	Vinyl	+	−	+	
Methyl	Ethyl	+	−	+	+
"	*n*-Butyl	+	−	+	+
"	*n*-Hexyl	+	−	+	+
Ethyl	*n*-Butyl	+	−	+	+
"	*n*-Hexyl	+	−	+	+
Propyl	*n*-Butyl	+	−	−	+
"	*n*-Amyl	+	−	−	+

In Table IV are given the configurational relationships of substances of Type I and of the corresponding α-substituted acids. The results of corresponding reactions of substitution are given in Table V.

From Table V it appears that substitution of a halogen atom for a hydroxyl group or of an amino group for a halogen atom

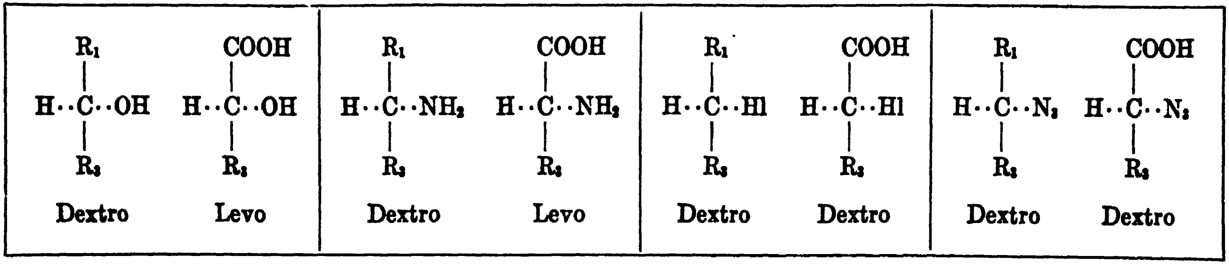
TABLE IV

Configurational Relationship of Substances



$$\begin{array}{c} R_1 \\ | \\ H\cdot\cdot C\cdot\cdot X \\ | \\ R_3 \end{array} \quad \text{and} \quad \begin{array}{c} COOH \\ | \\ H\cdot\cdot C\cdot\cdot X \\ | \\ R_3 \end{array}$$

(X = OH, Hl, NH_2; $R_1 < R_3$)*



$\begin{array}{c} R_1 \\ \vert \\ H\cdot\cdot C\cdot\cdot OH \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} COOH \\ \vert \\ H\cdot\cdot C\cdot\cdot OH \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} R_1 \\ \vert \\ H\cdot\cdot C\cdot\cdot NH_2 \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} COOH \\ \vert \\ H\cdot\cdot C\cdot\cdot NH_2 \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} R_1 \\ \vert \\ H\cdot\cdot C\cdot\cdot Hl \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} COOH \\ \vert \\ H\cdot\cdot C\cdot\cdot Hl \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} R_1 \\ \vert \\ H\cdot\cdot C\cdot\cdot N_3 \\ \vert \\ R_3 \end{array}$	$\begin{array}{c} COOH \\ \vert \\ H\cdot\cdot C\cdot\cdot N_3 \\ \vert \\ R_3 \end{array}$
Dextro	Levo	Dextro	Levo	Dextro	Dextro	Dextro	Dextro

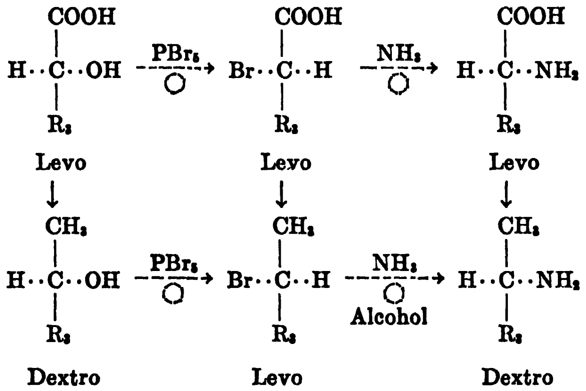
*The rotations of these substances are levo if $R_1 > R_3$.

proceeds similarly in the acids and in the alkyl derivatives. Furthermore, the fact that in the bromo and iodo derivatives the partial rotation of the halogen atom has the same sign in the acid and in the alkyl derivative permits the conclusion that the same relationship exists in the chloro derivatives. This is an important conclusion, because in the chloro acids the rotatory dispersion curve does not permit a definite decision as to which of the two polar groups furnishes the first partial rotation.

Vinyl Derivatives—In this series of unsaturated compounds two consecutive reactions of substitution bring about an inversion

TABLE V

Reactions of Substitution

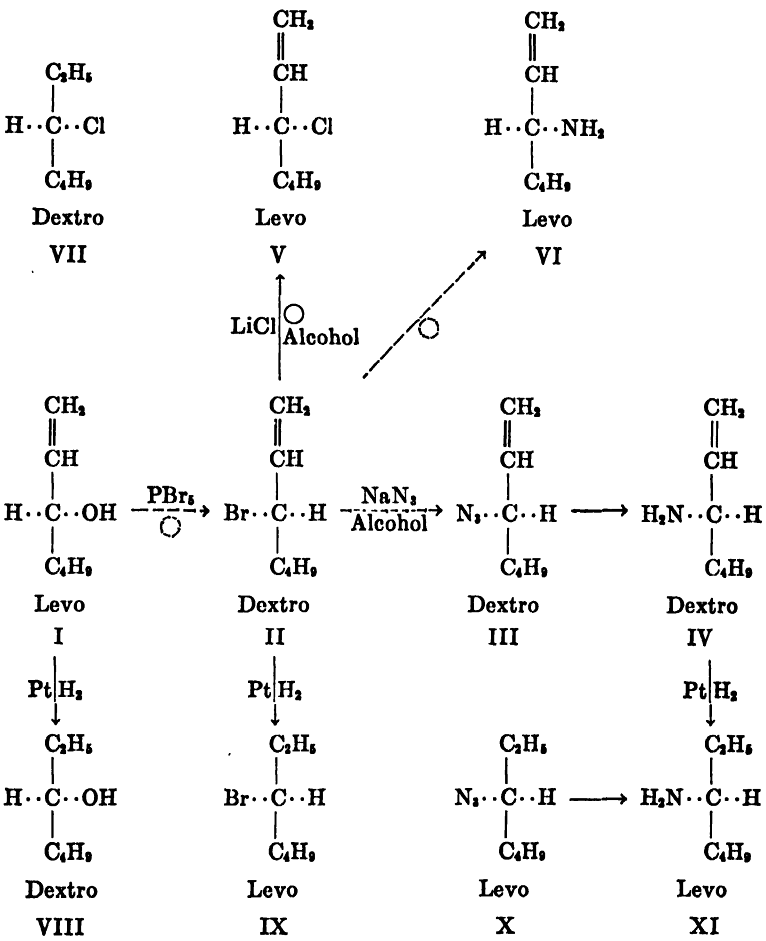


$$\begin{array}{ccccc}
\mathrm{COOH} & & \mathrm{COOH} & & \mathrm{COOH}\\
| & & | & & |\\
\mathrm{H\cdot\cdot C\cdot\cdot OH} & \xrightarrow[\bigcirc]{PBr_5} & \mathrm{Br\cdot\cdot C\cdot\cdot H} & \xrightarrow[\bigcirc]{NH_3} & \mathrm{H\cdot\cdot C\cdot\cdot NH_2}\\
| & & | & & |\\
R_3 & & R_3 & & R_3\\
\text{Levo} & & \text{Levo} & & \text{Levo}\\
\downarrow & & \downarrow & & \downarrow\\
CH_3 & & CH_3 & & CH_3\\
| & & | & & |\\
\mathrm{H\cdot\cdot C\cdot\cdot OH} & \xrightarrow[\bigcirc]{PBr_5} & \mathrm{Br\cdot\cdot C\cdot\cdot H} & \xrightarrow[\bigcirc\ \text{Alcohol}]{NH_3} & \mathrm{H\cdot\cdot C\cdot\cdot NH_2}\\
| & & | & & |\\
R_3 & & R_3 & & R_3\\
\text{Dextro} & & \text{Levo} & & \text{Dextro}
\end{array}$$

of configuration (see second row of Table VI). This conclusion is warranted, since the configurationally related saturated derivatives may be substituted for each vinyl derivative (see third row of Table VI). Substitution of a halogen atom for a hydroxyl group, of 1 halogen atom for another, or of an amino group for a halogen atom proceeds similarly in the two series of unsaturated and saturated compounds. Only the substitution of an azido group for a halogen atom is different in these two series. Thus it may be assumed that this last reaction of substitution proceeds without change of configuration in the vinyl derivatives. It is also evident that the molecular structure and not the external conditions are responsible for the difference in the outcome of this reaction, inasmuch as the conditions of the reaction were similar in both series.

TABLE VI

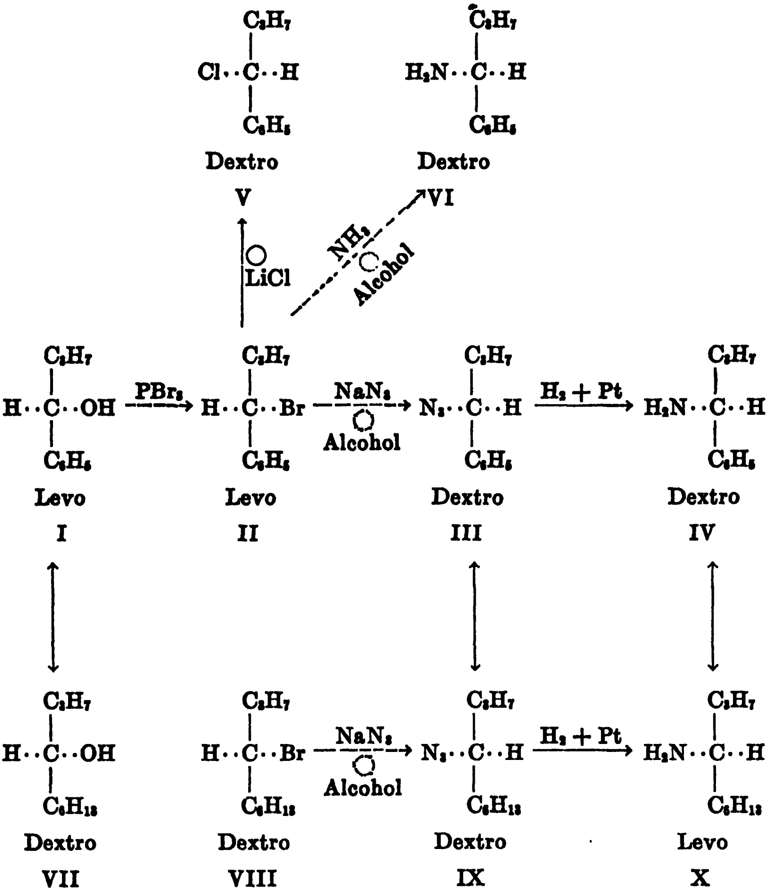
Reactions of Substitution in Unsaturated Derivatives



Alkylaryl Derivatives—In the group of substances given in Table VII two consecutive substitutions lead also to a change in configuration (see the second row), since the corresponding configurationally related alkyl derivatives can be substituted for all the alkylphenyl derivatives, except the halides. Furthermore, substitution of (1) 1 halogen atom for another, (2) an amino group for a halogen atom, (3) an azido group for a halogen atom proceeds with change of sign as in the alkyl series. In other words, the reaction of substi-

TABLE VII

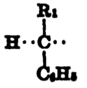
Reactions of Substitutions in Phenyl Derivatives



tution from (I) to (II) leads to substances correlated as are (VII) to (VIII); that is, in substance (I) the substitution of a halogen atom for a hydroxyl group proceeds without change of configuration. It must be mentioned that, if methyl or ethylphenyl carbinol is taken as the starting material, then the reactions of substitution follow a course analogous to that of the corresponding reaction in the alkyl series, as can be seen from Table VIII. Again it must be emphasized that the chloro derivatives of the ethylphenyl and of the propylphenyl carbinols are similar in their rotatory dispersion curves and hence are configurationally related, when they have the same sign of rotation.

TABLE VIII

Direction of Rotation of Derivatives Containing a Phenyl Group, the Bromides Being Prepared by the Action of HBr

 $\mathrm{H\cdot\cdot\overset{R_1}{\underset{C_6H_5}{C}}\cdot\cdot}$	$\cdot\cdot OH$	$\cdot\cdot Br$	$\cdot\cdot N_3$	$\cdot\cdot NH_2$
R_1				
Methyl	+	−	+	+
Ethyl	+	−	+	+
Propyl	+	+	−	−

SUMMARY

In summing up the results recorded in Tables I, VI, and VII it may be stated that:

1. In normal saturated aliphatic derivatives, substitution on the asymmetric carbon by a negative group or atom is connected with an inversion of configuration.

2. In substances of Type I, having R_1 = $—CH{=}CH_2$, substitution of an azido group for a halogen proceeds without inversion of configuration.

3. In substances of Type I, having R_3 = $—C_6H_5$, substitution of Br for a hydroxyl by HBr or PBr_3 in the absence of pyridine proceeds without inversion of configuration, in the presence of pyridine with inversion of configuration.

4. A formulation of the mechanism of the reaction of substitution on the asymmetric carbon atom is possible at this time for a

limited group of substances, namely for the normal saturated alkyl derivatives.

5. For this group of substances, the reaction of substitution is connected with an inversion of configuration, provided the mechanism of substitution of an N_3 group for halogen proceeds by the same mechanism as the substitution of one halogen for another.

6. A general formulation of the mechanism of the reaction of substitution is not yet possible. Consequently, a general theory of Walden inversion likewise is not yet possible.

EXPERIMENTAL

Dextro-4-Chlorooctane—10 gm. of 4-iodooctane, $\alpha_D^{25} = -2.00°$ (homogeneous, 1 dm.), were added to a solution of 9 gm. of lithium chloride in 135 cc. of methanol. This was placed in an oven at 37° for 1 week. The reaction mixture was poured into a concentrated calcium chloride solution and the halide was extracted with pentane. The extract was dried with phosphoric anhydride, and the chloride was distilled. B.p. 92°, $p = 50$ mm. Yield 2 gm. $\alpha_D^{25} = +0.28°$ (homogeneous, 1 dm.).

3.516 mg. substance: 8.373 mg. CO_2 and 3.715 mg. H_2O

$C_8H_{17}Cl$.	Calculated.	C 64.60, H 11.53
148.6	Found.	" 64.94, " 11.82

Dextro-3-Chlorononane—10 gm. of 3-iodononane, $\alpha_D^{25} = -18.2°$ (homogeneous, 1 dm.), were added to a solution of 9 gm. of lithium chloride in 180 cc. of methanol. This was placed in an oven at 37° for 6 days. (The reaction at room temperature was slow. After 3 days the iodide was recovered and its rotation was −16.2°.) The halide was isolated as usual. B.p. 98°, $p = 33$ mm. Yield 3 gm. $\alpha_D^{25} = +5.10°$ (homogeneous, 1 dm.).

4.200 mg. substance: 10.210 mg. CO_2 and 4.410 mg. H_2O

$C_9H_{19}Cl$.	Calculated.	C 66.42, H 11.78
162.6	Found.	" 66.34, " 11.75

Levo-1-Hepten-3-ol (*Butylvinylcarbinol*)—The inactive carbinol was prepared and resolved as previously described.[4] The acid phthalic ester was, however, made by a slightly different procedure. The mixture of phthalic anhydride, pyridine, and carbinol was heated in a water bath kept at 75° for 3 hours. The subsequent procedure was unchanged. The insoluble strychnine salt, after recrystallization, yielded a carbinol which distilled at 103–106°, p = 147 mm.

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[4] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **83**, 579 (1929).

$$[\alpha]_D^{25} = \frac{-19.30°}{1 \times 0.833} = -23.2°; \qquad [M]_D^{25} = -26.5° \text{ (homogeneous)}$$

4.012 mg. substance: 10.835 mg. CO_2 and 4.440 mg. H_2O
$C_7H_{14}O$. Calculated. C 73.61, H 12.37
114.1 Found. " 73.64, " 12.38

Dextro-3-Chloro-1-Heptene—50 gm. of 1-hepten-3-ol, $[\alpha]_D^{25}$ = −23.2° (homogeneous), were treated with 110 gm. of phosphorus pentachloride in dry ether according to the procedure previously described.[5] The chloride distilled at 92–94°, p = 125 mm. Yield 44 gm.; d_4^{25} = 0.8857 (*in vacuo*).

$$[\alpha]_D^{25} = \frac{+8.65°}{1 \times 0.886} = +9.76°; \qquad [M]_D^{25} = +12.9° \text{ (homogeneous)}$$

3.410 mg. substance: 7.890 mg. CO_2 and 2.980 mg. H_2O
$C_7H_{13}Cl$. Calculated. C 63.37, H 9.88
132.6 Found. " 63.09, " 9.77

Levo-3-Azido-1-Heptene—44 gm. of 3-chloro-1-heptene, $[\alpha]_D^{25}$ = +9.76° (homogeneous), were added to a solution of 27 gm. of sodium azide (Kahlbaum) in 75 cc. of water and 530 cc. of methanol. The resulting solution was transferred into pressure bottles, and allowed to stand at room temperature (25°) for 1 week. An equal volume of concentrated calcium chloride solution was then added, and the azide was extracted with pentane. The extract was washed with concentrated calcium chloride solution and dried with anhydrous calcium sulfate. After removal of the solvent, the residue distilled at 78–81°, p = 32 mm. Yield 36 gm.; d_4^{27} = 0.888 (*in vacuo*).

$$[\alpha]_D^{27} = \frac{-0.14°}{4 \times 0.89} = -0.04°; \qquad [M]_D^{27} = -0.06° \text{ (homogeneous)}$$

3.275 mg. substance: 7.225 mg. CO_2 and 2.810 mg. H_2O
$C_7H_{13}N_3$. Calculated. C 60.37, H 9.42
139.1 Found. " 60.16, " 9.60

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[5] Levene, P. A., and Haller, H. L., *J. Biol. Chem.*, **81**, 425 (1929).

Levo-3-Amino-1-Heptene—140 gm. of 3-chloro-1-heptene, $[\alpha]_D^{25} = +8.86°$ (homogeneous), were treated with ammonia in the following manner. 5 gm. of the halide were dissolved in 40 cc. of a 40 per cent solution of ammonia in methanol (which was made by adding liquid ammonia to cooled methanol) and sealed in a bomb tube. This was allowed to stand at 25° for 1 day, and was then heated at 50° for 3 days. The tube was cooled, opened, and the solution poured into ice. Concentrated sodium hydroxide solution was added, and the amine was extracted with ether. The extract was washed with concentrated sodium hydroxide solution, and dried with solid sodium hydroxide. The ether and methanol were distilled off at atmospheric pressure. The primary amine distilled at 95–105°, $p = 155$ mm. Yield 31 gm. $\alpha_D^{25} = -4.80°$ (homogeneous, 1 dm.).

3.289 mg. substance: 8.910 mg. CO_2 and 3.950 mg. H_2O
$C_7H_{15}N$. Calculated. C 74.25, H 13.36
113.1 Found. " 73.87, " 13.43

The higher fractions contained secondary and tertiary amines. A pure secondary amine was obtained from a chloride of $[\alpha]_D^{25} = -6.2°$ (homogeneous). B.p. 92–95°, $p = 1$ mm. $\alpha_D^{25} = +0.52°$ (homogeneous, 1 dm.).

3.002 mg. substance: 8.880 mg. CO_2 and 3.514 mg. H_2O
$C_{14}H_{27}N$. Calculated. C 80.29, H 13.01
209.24 Found. " 80.66, " 13.10

Levo-3-Aminoheptane—30 gm. of 3-azido-1-heptene, $[\alpha]_D^{27} = -0.04°$ (homogeneous), were dissolved in 70 cc. of methanol, and 1.4 gm. of Adams' catalyst were added. This was shaken (in two portions) overnight with hydrogen at a pressure of 3 atmospheres. The catalyst was then filtered off, and hydrogen chloride in methanol was added to the filtrate. This was evaporated to dryness under reduced pressure. The crystals were taken up in 50 per cent potassium hydroxide and extracted with ether. The extract was dried over metallic sodium. The amine distilled at 100–106°, $p = 148$ mm.

$$[\alpha]_D^{27} = \frac{-0.016°}{1 \times 0.783} = -0.02°; \qquad [M]_D^{27} = -0.02° \text{ (homogeneous)}$$

4.221 mg. substance: 11.340 mg. CO_2 and 5.525 mg. H_2O
$C_7H_{17}N$. Calculated. C 72.95, H 14.88
115.1 Found. " 73.26, " 14.64

Levo-3-Aminoheptane—10 gm. of 3-amino-1-heptene, $\alpha_D^{25} = +0.8°$ (homogeneous, 1 dm.), were dissolved in 25 cc. of methanol and 0.5 gm. of Adams' catalyst was added. This was shaken with hydrogen at a pressure of 3 atmospheres overnight. The free amine was isolated as described above. B.p. 99–101°, p = 150 mm.

$$[\alpha]_D^{27} = \frac{-0.35°}{1 \times 0.783} = -0.45°; \quad [M]_D^{27} = -0.52° \text{ (homogeneous)}$$

4.159 mg. substance: 11.090 mg. CO_2 and 5.490 mg. H_2O

$C_7H_{17}N$.	Calculated.	C 72.95, H 14.88
115.1	Found.	" 72.71, " 14.77

Levo-3-Bromo-1-Heptene—20 gm. of 1-hepten-3-ol, $[\alpha]_D^{25} = +10.5°$ (homogeneous), were dissolved in 30 cc. of dry ether, and added dropwise into a cooled suspension of 105 gm. of phosphorus pentabromide in 200 cc. of dry ether, with stirring. After the carbinol was added, the solution was allowed to stand for 1 hour at room temperature. The bromide was isolated as usual.[5] B.p. 92–94°, p = 50 mm. Yield 10 gm.; d_4^{25} = 1.164 (*in vacuo*).

$$[\alpha]_D^{25} = \frac{-5.40°}{1 \times 1.164} = -4.64°; \quad [M]_D^{25} = -8.22° \text{ (homogeneous)}$$

This rotation remained unchanged after 6 weeks standing at a temperature of about 5°.

3.214 mg. substance: 5.600 mg. CO_2 and 2.170 mg. H_2O

$C_7H_{13}Br$.	Calculated.	C 47.45, H 7.40
177.0	Found.	" 47.51, " 7.55

Dextro-3-Chloro-1-Heptene—8.5 gm. of 3-bromo-1-heptene, $\alpha_D^{25} = -5.40°$ (homogeneous, 1 dm.), were added to a solution of 11 gm. of dry lithium chloride in 100 cc. of methanol (the warm solution of the salt was cooled before the bromide was added) and let stand at room temperature (about 25°) for 10 days. The solution was then poured into concentrated calcium chloride solution and extracted with pentane. The extract was washed with concentrated calcium chloride solution and dried with phosphoric anhydride overnight. After filtration and removal of the solvent, the residue was distilled. B.p. 87–88°, p = 90 mm. Yield 2 gm.

$$[\alpha]_D^{25} = \frac{+0.80^\circ}{1 \times 0.89} = +0.90^\circ; \qquad [M]_D^{25} = +1.19^\circ \text{ (homogeneous)}$$

5.024 mg. substance: 11.700 mg. CO_2 and 4.410 mg. H_2O
5.012 " " : 5.425 " AgCl
$C_7H_{13}Cl$. Calculated. C 63.37, H 9.88, Cl 26.75
132.6 Found. " 63.50, " 9.82, " 26.77

Levo-1-Phenyl-1-Ethanol (*Methylphenylcarbinol*)[6]—The inactive carbinol was prepared by a Grignard reaction with bromobenzene and acetaldehyde. The carbinol was converted into the acid phthalic ester with pyridine. The phthalate was recrystallized from a mixture of ether and pentane. The pure phthalate melted at 108°. 1080 gm. of the phthalate were converted into the brucine salt in acetone and the salt recrystallized from 95 per cent acetone fifteen times. The phthalate, which was recovered from the original mother liquor, partially crystallized on standing. The crystals were filtered and on hydrolysis gave 16 gm. of a carbinol with a rotation of $\alpha_D^{25} = +1.3^\circ$ (homogeneous, 1 dm.). The filtrate gave 20 gm. of a carbinol with a rotation of $\alpha_D^{25} = +30^\circ$. Mother Liquors 2 to 8 were combined and the phthalate recovered. The crystalline phthalate gave 58 gm. of carbinol, $\alpha_D^{25} = +2.3^\circ$, and the filtrate yielded 62 gm. of carbinol, $\alpha_D^{25} = +37^\circ$. Mother Liquors 8 to 12 were treated the same way. The crystalline phthalate gave 54 gm. of carbinol, $\alpha_D^{25} = -0.5^\circ$, and the filtrate yielded 17 gm. of carbinol, $\alpha_D^{25} = -30^\circ$.

The insoluble brucine salt yielded 35 gm. of carbinol. B.p. 75°, p = 1 mm.

$$[\alpha]_D^{25} = \frac{-42.0^\circ}{1 \times 1.00} = -42.0^\circ; \qquad [M]_D^{25} = -51.3^\circ \text{ (homogeneous)}$$

4.377 mg. substance: 12.585 mg. CO_2 and 3.210 mg. H_2O
$C_8H_{10}O$. Calculated. C 78.64, H 8.26
122.1 Found. " 78.40, " 8.20

Levo-1-Chloro-1-Phenylethane (*Methylphenylchloromethane*)[7]—38 gm. of 1-phenyl-1-ethanol, $[\alpha]_D^{25} = -36.3^\circ$ (homogeneous), were dissolved in 50 gm. of redistilled thionyl chloride with cooling. The solution was allowed to stand at room temperature for 10 minutes and then was refluxed for 15 minutes. The reaction mixture was poured into ice and the chloride was extracted with pentane. The extract was washed with water and dried with phosphoric anhydride. The chloride distilled at 101°, $p = 50$ mm. Yield 39 gm.

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[6] Levene, P. A., and Mikeska, L. A., *J. Biol. Chem.*, **70**, 355 (1926).

[7] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **99**, 71 (1911).

$$[\alpha]_{\mathrm{D}}^{25} = \frac{-25.2^\circ}{1 \times 1.05} = -24.0^\circ; \quad [M]_{\mathrm{D}}^{25} = -33.7^\circ \text{ (homogeneous)}$$

4.734 mg. substance: 11.900 mg. CO_2 and 2.790 mg. H_2O
C_8H_9Cl. Calculated. C 68.31, H 6.45
140.5 Found. " 68.54, " 6.59

Dextro-1-Azido-1-Phenylethane (*Methylphenylazidomethane*)—39 gm. of 1-chloro-1-phenylethane, $[\alpha]_{\mathrm{D}}^{25} = -24.0^\circ$ (homogeneous), were added to a solution of 27 gm. of sodium azide (Kahlbaum) in 70 cc. of water and 475 cc. of methanol. The solution was refluxed for 5 hours. The azide was isolated as usual. B.p. 114°, $p = 50$ mm. Yield 22 gm.; $d_4^{25} = 1.0321$ (*in vacuo*); $n_{\mathrm{D}}^{25} = 1.5233$.

$$[\alpha]_{\mathrm{D}}^{25} = \frac{+19.2^\circ}{1 \times 1.03} = +18.6^\circ; \quad [M]_{\mathrm{D}}^{25} = +27.4^\circ \text{ (homogeneous)}$$

5.410 mg. substance: 13.005 mg. CO_2 and 3.100 mg. H_2O
$C_8H_9N_3$. Calculated. C 65.27, H 6.17
147.1 Found. " 65.55, " 6.41

Dextro-1-Amino-1-Phenylethane (*Methylphenylaminomethane*)—20 gm. of 1-azido-1-phenylethane, $[\alpha]_{\mathrm{D}}^{25} = +18.6^\circ$ (homogeneous), were dissolved in 50 cc. of methanol, 0.6 gm. of Adams' catalyst was added, and the mixture was shaken with hydrogen at a pressure of 3 atmospheres for 5 hours. The hydrochloride and the free base were isolated as usual. The amine boiled at 75°, $p = 15$ mm. Yield 5 gm.

$$[\alpha]_{\mathrm{D}}^{25} = \frac{+2.98^\circ}{1 \times 0.95} = +3.14^\circ; \quad [M]_{\mathrm{D}}^{25} = +3.80^\circ \text{ (homogeneous)}$$

3.992 mg. substance: 11.605 mg. CO_2 and 3.220 mg. H_2O
$C_8H_{11}N$. Calculated. C 79.27, H 9.16
121.1 Found. " 79.27, " 9.03

Levo-1-Phenyl-1-Propanol (*Ethylphenylcarbinol*)[6]—The inactive carbinol was made from ethyl magnesium bromide and benzaldehyde. It was converted into the acid phthalic ester with pyridine. The phthalate was converted into the strychnine salt which was recrystallized from ethyl acetate four times. The crystals yielded 85 gm. of carbinol. B.p. 94–95°, $p = 10$ mm.

$$[\alpha]_D^{25} = \frac{-22.0^\circ}{1 \times 0.99} = -22.2^\circ; \qquad [M]_D^{25} = -30.2^\circ \text{ (homogeneous)}$$

3.024 mg. substance: 8.805 mg. CO_2 and 2.410 mg. CO_2

$C_9H_{12}O$.	Calculated.	C 79.35, H 8.89
136.1	Found.	" 79.40, " 8.91

Levo-1-Chloro-1-Phenylpropane (*Ethylphenylchloromethane*)[6, 8]—75 gm. of 1-phenyl-1-propanol, $[\alpha]_D^{25} = -22.2^\circ$ (homogeneous), were cooled in an ice-alcohol bath, and 110 gm. of freshly distilled thionyl chloride were slowly added. The solution was allowed to stand at room temperature for 30 minutes and then was refluxed for 15 minutes. The chloride was isolated as usual. B.p. 77–80° $p = 10$ mm. Yield 57 gm.

$$[\alpha]_D^{25} = \frac{-29.8^\circ}{1 \times 1.03} = -28.9^\circ; \qquad [M]_D^{25} = -44.7^\circ \text{ (homogeneous)}$$

3.514 mg. substance: 9.010 mg. CO_2 and 2.270 mg. H_2O

$C_9H_{11}Cl$.	Calculated.	C 69.88, H 7.18
154.6	Found.	" 69.92, " 7.22

Levo-1-Bromo-1-Phenylpropane (*Ethylphenylbromomethane*)— 30 gm. of 1-phenyl-*1*-propanol, $[\alpha]_D^{25} = +13.1^\circ$ (homogeneous), were dissolved in 24 gm. of dry pyridine, and slowly added to a cooled suspension of 90 gm. of phosphorus pentabromide in 150 cc. of chloroform. The reaction mixture was then allowed to stand at 25° for 4 hours. The bromide was isolated as usual. B. p. 57–61°, $p = 0.6$ mm. Yield 24 gm.

$$\alpha_D^{25} = -47.7^\circ \text{ (homogeneous)}$$

5.994 mg. substance: 11.915 mg. CO_2 and 3.010 mg. H_2O

$C_9H_{11}Br$.	Calculated.	C 54.27, H 5.57
199.0	Found.	" 54.20, " 5.61

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[8] Pickard, R. H., and Kenyon, J., *J. Chem. Soc.*, **99**, 69 (1911).

Dextro-1-Azido-1-Phenylpropane (Ethylphenylazidomethane)—57 gm. of 1-chloro-1-phenylpropane, $[\alpha]^{25}_D = -28.9°$ (homogeneous), were added to a solution of 40 gm. of sodium azide in 130 cc. of water and 730 cc. of methanol. The solution was refluxed for 16 hours. The azide was isolated as usual. B.p. 100–101°, $p = 22$ mm. Yield 30 gm. $\alpha^{25}_D = +32.95°$ (homogeneous, 1 dm.).

3.091 mg. substance: 7.604 mg. CO_2 and 1.930 mg. H_2O
$C_9H_{11}N_3$. Calculated. C 67.03, H 6.88
161.1 Found. " 67.08, " 6.98

Dextro-1-Amino-1-Phenylpropane (Ethylphenylaminomethane)—27 gm. of 1-azido-1-phenylpropane, $\alpha^{25}_D = +32.95°$ (homogeneous, 1 dm.), were dissolved in 70 cc. of methanol and 1.5 gm. of Adams' catalyst were added. The mixture was shaken with hydrogen at a pressure of 3 atmospheres for 5 hours. The hydrochloride and the free base were isolated as usual. The amine boiled at 81°, $p \doteq 10$ mm. Yield 14 gm.

$$[\alpha]^{25}_D = \frac{+4.25°}{1 \times 0.93} = +4.57°; \quad [M]^{25}_D = +6.17° \text{ (homogeneous)}$$

4.604 mg. substance: 13.490 mg. CO_2 and 3.990 mg. H_2O
$C_9H_{13}N$. Calculated. C 79.93, H 9.70
135.1 Found. " 79.90, " 9.69

Dextro-1-Chloro-1-Phenylpropane—8 gm. of 1-bromo-1-phenylpropane, $\alpha^{25}_D = -8.04°$ (homogeneous, 1 dm.), prepared according to Levene and Mikeska,[6] were added to a cooled solution of 9 gm. of lithium chloride in 82 cc. of methanol. This was allowed to stand at room temperature for 3 days. The halide was isolated as described above. B.p. 87–89°, $p = 18$ mm. Yield 2 gm. $\alpha^{25}_D = +2.05°$ (homogeneous, 1 dm.).

4.002 mg. substance: 10.245 mg. CO_2 and 2.640 mg. H_2O
$C_9H_{11}Cl$. Calculated. C 69.88, H 7.18
154.6 Found. " 69.81, " 7.38

Dextro-1-Amino-1-Phenylpropane—24 gm. of 1-bromo-1-phenylpropane, $\alpha^{25}_D = -47.7°$ (homogeneous, 1 dm.), were dissolved in 300 cc. of a 40 per cent solution of ammonia in methanol (made by adding liquid ammonia to cooled methanol). The solution was placed in two magnesia bottles and allowed to stand at 25° for 4 days. The bottles were then cooled and opened. The solution was slowly dropped into 250 cc. of concentrated aqueous hydrochloric acid which was cooled in a freezing mixture. The amine was isolated as described for the 3-amino-1-heptene. B.p. 88–90°, $p = 16$ mm. Yield 6 gm.

$$[\alpha]_D^{25} = \frac{+3.39°}{1 \times 0.93} = +3.65°; \qquad [M]_D^{25} = +4.93° \text{ (homogeneous)}$$

3.878 mg. substance: 11.365 mg. CO_2 and 3.325 mg. H_2O
$C_9H_{13}N$. Calculated. C 79.93, H 9.70
135.1 Found. " 79.91, " 9.59

Levo-1-Phenyl-1-Butanol (*Propylphenylcarbinol*)[6,9]—The inactive carbinol was resolved by recrystallization of the strychnine salt of the acid phthalic ester. A carbinol was obtained from the crystals which boiled at 121–123°, $p = 18$ mm. The carbinol crystallized. The rotation of a melted portion was determined.

$$[\alpha]_D^{25} = \frac{-7.70°}{1 \times 1.01} = -7.62°; \qquad [M]_D^{25} = -11.4° \text{ (homogeneous)}$$

3.625 mg. substance: 10.625 mg. CO_2 and 3.090 mg. H_2O
$C_{10}H_{14}O$. Calculated. C 79.94, H 9.40
150.1 Found. " 79.92, " 9.53

Levo-1-Cyclohexyl-1-Butanol (*Propylcyclohexylcarbinol*)[9]—10 gm. of 1-phenyl-1-butanol, $[\alpha]_D^{25} = -7.62°$ (homogeneous), were dissolved in 25 cc. of a solution containing 90 gm. of methanol and 10 gm. of glacial acetic acid,[10] and 0.5 gm. of Adams' catalyst was added. The mixture was shaken with hydrogen at a pressure of 3 atmospheres for 16 hours. The pressure change was 21 pounds (Burgess Parr Company, Illinois, model apparatus). The catalyst was filtered off, and the filtrate was distilled. The carbinol boiled at 76–77°, $p = 1.5$ mm. Yield 6.5 gm.

$$[\alpha]_D^{25} = \frac{-4.15°}{1 \times 0.90} = -4.61°; \qquad [M]_D^{25} = -7.20° \text{ (homogeneous)}$$

5.096 mg. substance: 14.390 mg. CO_2 and 5.815 mg. H_2O
$C_{10}H_{20}O$. Calculated. C 76.84, H 12.91
156.2 Found. " 77.00, " 12.77

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[9] Levene, P. A., and Marker, R. E., *J. Biol. Chem.*, **97**, 379 (1932).

[10] Levene, P. A., *J. Biol. Chem.*, **115**, 275 (1936).

Dextro-1-Bromo-1-Phenylbutane (from Levo-1-Phenyl-1-Butanol) —27 gm. of 1-phenyl-1-butanol, $[\alpha]^{25}_{D} = -7.62°$ (homogeneous), were dissolved in 18 gm. of dry pyridine, and then slowly added to a cooled suspension of 75 gm. of phosphorus pentabromide (Kahlbaum) in 75 cc. of dry chloroform. The mixture was allowed to stand at room temperature with occasional shaking for 3 hours. (All of the phosphorus pentabromide did not react.) The bromide was isolated as usual. The pentane extract was dried over phosphoric anhydride. The bromide distilled at 67–75°, $p = 0.5$ to 1 mm. Yield 33 gm.; $d^{25}_{4} = 1.2631$ (*in vacuo*); $n^{25}_{D} = 1.5421$.

$$[\alpha]^{25}_{D} = \frac{+22.2°}{1 \times 1.26} = +17.6°; \qquad [M]^{25}_{D} = +37.5° \text{ (homogeneous)}$$

4.615 mg. substance: 9.515 mg. CO_2 and 2.500 mg. H_2O
$C_{10}H_{13}Br$. Calculated. C 56.33, H 6.15
213.0 Found. " 56.22, " 6.06

Levo-1-Bromo-1-Phenylbutane (from Levo-1-Phenyl-1-Butanol)[6]— 8.5 gm. of 1-phenyl-1-butanol, $[\alpha]^{25}_{4} = -7.48°$ (homogeneous), were added to a cooled suspension of 25 gm. of phosphorus pentabromide (Kahlbaum) in 25 cc. of dry chloroform. The mixture was allowed to stand at room temperature for 3 hours. (All of the phosphorus pentabromide dissolved.) The bromide was isolated as usual. B.p. 67–72°, $p = 0.5$ to 1 mm. Yield 8 gm.

$$[\alpha]^{25}_{D} = \frac{-2.50°}{1 \times 1.26} = -1.98°; \qquad [M]^{25}_{D} = -4.22° \text{ (homogeneous)}$$

30 gm. of 1-phenyl-1-butanol, $[\alpha]^{25}_{D} = -7.48°$ (homogeneous), were cooled, and 60 gm. of phosphorus tribromide were slowly added. The solution was heated on the steam bath for 45 minutes. The bromide was isolated as usual. B.p. 65–72°, $p = 0.5$ to 1 mm. Yield 38 gm.

$$[\alpha]^{25}_{D} = \frac{-0.25°}{1 \times 1.26} = -0.20°; \qquad [M]^{25}_{D} = -0.42° \text{ (homogeneous)}$$

When phosphorus pentachloride was used with the pyridine solution under the same conditions, the chloride decomposed completely before distillation. A hydrocarbon which boiled at 147–152°, $p = 1.5$ mm., was obtained. Analysis showed the composition to be $C_{10}H_{12}$.

Levo-1-Chloro-1-Phenylbutane—7.5 gm. of 1-bromo-1-phenylbutane, $\alpha^{25}_{D} = +18.0°$ (homogeneous, 1 dm.), were added to a solution of 9 gm. of lithium chloride in 130 cc. of methanol. The resulting solution was allowed to stand at 50° for 36 hours. The halide was isolated as usual. A mixture of hydrocarbon, chloride, and unchanged bromide was obtained. The fraction corresponding to the chloride distilled at 119–128°, $p = 37$ mm., and had a rotation of $\alpha^{25}_{D} = -2.70°$ (homogeneous, 1 dm.).

4.204 mg. substance: 10.820 mg. CO_2 and 2.715 mg. H_2O
5.282 " " : 4.805 " AgCl

$C_{10}H_{13}Cl$.	Calculated.	C 71.19, H 7.77, Cl 21.04
168.6	Found.	" 70.18, " 7.22, " 22.56

Levo-1-Azido-1-Phenylbutane (*Propylphenylazidomethane*)—12 gm. of 1-bromo-1-phenylbutane, $[\alpha]^{25}_{D} = +10.7°$ (homogeneous), were added to a solution of 12 gm. of sodium azide in 30 cc. of water and 200 cc. of methanol. The solution was allowed to stand for 16 hours at 25°. The azide was isolated as usual. B.p. 81–87°, $p = 3$ mm. Yield 9 gm.; $d^{25}_{4} = 0.9893$ (*in vacuo*); $n^{25}_{D} = 1.5122$.

$$[\alpha]^{25}_{D} = \frac{-9.01°}{1 \times 0.989} = -9.11°; \quad [M]^{25}_{D} = -16.0° \text{ (homogeneous)}$$

30 gm. of 1-bromo-1-phenylbutane, $[\alpha]^{25}_{D} = +17.6°$ (homogeneous), yielded 18 gm. of an azide which boiled at 85–90°, $p = 4$ mm.

$$[\alpha]^{25}_{D} = \frac{-15.2°}{1 \times 0.989} = -15.4°; \quad [M]^{25}_{D} = -27.0° \text{ (homogeneous)}$$

4.630 mg. substance: 11.695 mg. CO_2 and 3.155 mg. H_2O

$C_{10}H_{13}N_3$.	Calculated.	C 68.52, H 7.48
175.1	Found.	" 68.88, " 7.62

Levo-1-Amino-1-Phenylbutane—27 gm. of 1-azido-1-phenylbutane, $[\alpha]^{25}_{D} = -13.2°$ (homogeneous), were dissolved in 50 cc. of methanol, and 1 gm. of Adams' catalyst was added. The mixture was shaken with hydrogen at a pressure of 3 atmospheres for 5 hours. The hydrochloride was isolated as usual.

$$[\alpha]^{25}_{D} = \frac{+0.14° \times 100}{2 \times 10.6} = +0.66°; \quad [M]^{25}_{D} = +1.22° \text{ (in water)}$$

The free amine was isolated and distilled at 105°, $p = 10$ mm. Yield 15 gm.; $d_4^{25} = 0.9197$ (*in vacuo*); $n_D^{25} = 1.5089$.

$$[\alpha]_D^{25} = \frac{-2.10^\circ}{1 \times 0.92} = -2.28^\circ; \qquad [M]_D^{25} = -3.40^\circ \text{ (homogeneous)}$$

3.802 mg. substance: 11.200 mg. CO_2 and 8.420 mg. H_2O

$C_{10}H_{15}N$.	Calculated.	C 80.47, H 10.14
149.1	Found.	" 80.33, " 10.06

Levo-1-Amino-1-Phenylbutane—27 gm. of 1-bromo-1-phenylbutane, $\alpha_D^{25} = +8.80^\circ$ (homogeneous), were dissolved in 300 cc. of 40 per cent ammonia in methanol and treated exactly as described for the 1-amino-1-phenylpropane. The amine distilled at 103–104°, $p = 15$ mm. Yield 6 gm.

$$[\alpha]_D^{25} = \frac{-0.76^\circ}{1 \times 0.92} = -0.83^\circ; \qquad [M]_D^{25} = -1.24^\circ \text{ (homogeneous)}$$

3.929 mg. substance: 11.604 mg. CO_2 and 3.565 mg. H_2O

$C_{10}H_{15}N$.	Calculated.	C 80.47, H 10.14
149.1	Found.	" 80.54, " 10.15

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ON LYSOLECITHIN AND ON TOSYLGLYCERIDES

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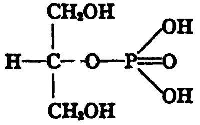
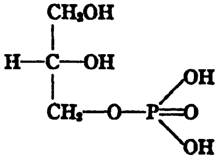
(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(July 5, 1937)

The object of the present investigation was to inquire into the structure of α-lysolecithins. The structures of β-lecithins and β-lysolecithins present no problem, for being derivatives of β-glycerophosphoric acid (I), both fatty acids in lecithins occupy terminal positions.



I



II

Hence, β-lysolecithins can have only one structure. Different is the case of α-lysolecithins, in which the saturated fatty acid may be attached to the primary or to the secondary carbon atom (II).

The lysolecithin in our possession had been prepared by the action of cobra venom on egg yolk; hence it was not certain whether it originated from the α- or β-lecithin, or from both, for its source could not be predicted either by analogy or by any theory. The first task was to discover the nature of the glycerophosphoric acid derived from our lysolecithin (1).

The lysolecithin was hydrolyzed by an aqueous solution of barium hydroxide at room temperature. By means of a double hydrolysis (conditions are given in the experimental part) the yield of glycerophosphoric acid was about 75 per cent of that required by theory.

The crude barium glycerophosphate, after purification by reprecipitation, was fractionated by the method of Karrer and Salomon (2).

The β-form was found to constitute over 86 per cent of the total.

Theoretically the determination of the position of the fatty acids in α-lysolecithins was not expected to offer any difficulty, inasmuch as primary and secondary alcoholic groups are known to possess distinct properties. Thus, in glycerophosphates, only the secondary and not the primary alcoholic groups are oxidized by bromine-water and on this property Bailly (3) based a method for differentiating α- and β-glycerophosphoric acids. We have confirmed Bailly's statement and furthermore have shown that α-methyl but not β-methylglycerol is oxidized appreciably by bromine-water under the conditions given by Bailly. Applied to lysolecithin, treatment with bromine-water with subsequent acidulation and distillation led to the formation of methylglyoxal. This could not have originated from the β-lysolecithin and therefore might have been interpreted as meaning that in α-lysolecithin the secondary alcoholic group was not substituted. However, a control experiment with α, β-distearylglycerol likewise resulted in the formation of methylglyoxal on distillation in the presence of sulfuric acid. Thus treatment with bromine-water brings about deacylation, and hence the method is inapplicable in the case of lysolecithin.

A second method of differentiation between primary and secondary alcoholic groups is based on the difference in the stabilities of their tosyl derivatives towards sodium iodide (4). In the primary derivatives, as a rule, the tosyl group is readily replaced, leading to an iodide but in the secondary the process is a very slow one, if it occurs at all. Preliminary to applying this method to lysolecithin, it was tested on glycerol derivatives of known structure. Hence tritosyl-glycerol and ditosyl-α-methylglycerol were prepared. Contrary to expectation it was found that the tosyl group was removed with equal ease from the primary and from the secondary carbon atoms.

A third method theoretically seemed available for the solution of our problem, namely, methylation, inasmuch as only α-lysolecithin having the acyl group in the terminal position could yield β-methylglycerol. However, this method was found inapplicable to lysolecithin for the reason that methylation by Purdie's method leads to saponification of the acyl groups.

Although our experiments failed to elucidate the position of the fatty acid residue in α-lysolecithin, they are presented here in order to record some peculiar properties of α-lysolecithin which it has in common with other acylated glycerols.

EXPERIMENTAL

Hydrolysis of Lysolecithin. 10 g of lysolecithin were emulsified in 50 cc of 95 per cent alcohol and 100 cc of water were used to remove the residual substance from the mortar and pestle. After the addition of 250 cc of saturated aqueous barium hydroxide solution (3 moles) the whole was shaken for 18 hours at room temperature and then filtered. The buff-colored residue, consisting of the barium salts of the fatty acids, was washed several times with water and the combined washings and filtrate carbonated and filtered to remove excess barium. The resulting filtrate was evaporated to dryness under diminished pressure at 40° and the residual barium carbonate removed by filtration after agitation with 300 cc of cold water. This filtrate was evaporated in the manner previously mentioned, to about 40 cc and several volumes of 95 per cent ethyl alcohol added to precipitate the barium glycerophosphate. The product was dissolved in 20 cc of water and re-precipitated with alcohol. After centrifuging and washing with alcohol and ether the substance was dried overnight under low vacuum over phosphorus pentoxide and weighed 2,56 g (42,6 per cent of the theoretical yield).

The substance had the following composition. The sample was dried to constant weight at 138° at low vacuum over sulfuric acid.

7,175 mg substance: 3,405 mg CO_2 and 1,785 mg H_2O
5,090 " " : 35,315 " ammonium phosphomolybdate
25,195 " " : 18,205 " $BaSO_4$
8,115 " " : 0,045 cc N_2 (751 mm at 28°)

$C_3H_7O_6PBa$	Calculated.	C 11,71	H 2,29	P 10,10	Ba 44,68	N 0,00
	Found.	" 12,93	" 2,78	" 10,07	" 42,52	" 0,00

Its specific rotation was

$$[\alpha]_D^{24} = \frac{-0,14^\circ \times 100}{2 \times 20,94} = -0,33^\circ \text{ (in water)}$$

To purify the barium glycerophosphate further, it was dissolved in a minimum of water and an excess of neutral lead acetate solution added. The precipitated lead glycerophosphate was washed free of soluble salts and a suspension of it in distilled water was treated with hydrogen sulfide. After removal of the dissolved hydrogen sulfide by a stream of air and the lead sulfide by filtration, the clear filtrate was neutralized with a slight excess of barium hydroxide solution and several volumes of alcohol added. The precipitated barium glycerophosphate was dried for 2 days over phosphorus pentoxide under a low vacuum. 5,59 per cent water was present. (5,53 per cent theoretical for 1 molecule of water of crystallization).

The composition and rotation were as follows:

Sample dried at 138° at low vacuum over sulfuric acid.

4,525 mg substance: 35,710 mg ammonium phosphomolybdate
12,170 " " : 8,705 " barium sulfate

No nitrogen was found.

$C_3H_7O_6PBa$	Calculated.	Ba 44,68	P 10,10	N 0,00
	Found.	" 42,09	" 9,81	" 0,00

$$[\alpha]_D^{28} = \frac{-0,19^\circ \times 100}{2 \times 20,10} = -0,47^\circ \text{ (in water)}$$

The barium fatty acid salts were dried and weighed 6,74 g (Theoretical yield calculated as barium stearate 6,85 g). To remove occluded phosphorus-containing compounds the residue was suspended in 100 cc of water and hydrolyzed with 100 cc of saturated aqueous barium hydroxide solution by shaking for 24 hours at room temperature. The reaction product was worked up in the usual manner and only 0,02 g of barium glycerophosphate was obtained.

The water-alcohol filtrate from the main barium glycerophosphate precipitation gave a positive phosphorus test and was then hydrolyzed with 100 cc. of saturated barium hydroxide solution for 22 hours at room temperature. On addition of alcohol to the concentrated water solution of barium glycerophosphate only 0,04 g of the barium salt was obtained. It appeared likely that some of the glycerophosphate was being held in solution as alkali salts when the barium glycerophosphate was being precipitated out with alcohol.

To recover these soluble salts the alcohol-water filtrate from the barium glycerophosphate precipitation was evaporated at low pressure and 40° to a small volume. Neutral lead acetate solution was added in excess and the white lead glycerophosphate that precipitated out was immediately converted into the barium salt in the usual way. 1,89 g were obtained. After drying overnight under a low vacuum over phosphorus pentoxide it contained 7,9 per cent moisture.

It had the following composition and rotation:

Sample dried at 138° under a low vacuum over sulfuric acid.

22,310 mg substance: 16,200 mg $BaSO_4$
3,600 " " : 25,040 " ammonium phosphomolybdate

No nitrogen was found.

$C_3H_7O_6PBa$				
	Calculated.	Ba 44,68	P 10,10	N 0,00
	Found.	" 42,73	" 10,10	" 0,00

$$[\alpha]_D^{24} = \frac{-0{,}28^\circ \times 100}{2 \times 20{,}\,06} = -0{,}70^\circ \text{ (in water)}$$

The product was purified further by converting it to the lead salt again and then back to barium glycerophosphate.

Its rotation was:

$$[\alpha]_D^{23} = \frac{-0{,}26^\circ \times 100}{2 \times 20{,}04} = -0{,}65^\circ \text{ (in water)}$$

The filtrate from the first lead glycerophosphate precipitation was hydrolyzed with saturated barium hydroxide solution but yielded no more barium glycerophosphate.

The total recovery of barium glycerophosphate was 75 per cent of the theoretical.

All of the barium glycerophosphate products were combined and precipitated twice through the lead salt and finally once with alcohol as the barium salt. The product was dried over phosphorus pentoxide at low vacuum for 66 hours. 5,10 per cent water was present as one molecule of water of crystallization. (Theory 5,53 per cent).

The composition of the product was the following:

The sample was dried at 138° and low vacuum over sulfuric acid.

39,400 mg substance: 29,530 mg $BaSO_4$
4,000 " " : 28,670 " ammonium phosphomolybdate

$C_3H_7O_6PBa$.			
	Calculated.	Ba 44,68	P 10,10
	Found.	" 44,09	" 10,40

Its rotation was

$$[\alpha]_D^{24} = \frac{-0{,}26° \times 100}{2 \times 20{,}20} = -0{,}64° \text{ (in water)}$$

Separation of Barium-β-Glycerophosphate from Barium-α-Glycerophosphate. 2,8757 g of barium-glycerophosphate from the hydrolysis of lysolecithin and containing 5,10 per cent water (the β-compound with 1 molecule of water of crystallization contains 5,53 per cent water) was dissolved in 28,80 cc of distilled water and 2,24 g of barium nitrate in 28,80 cc of distilled water added. The solution stood for 20 hours at room temperature and was then filtered from the insoluble double salt $(C_3H_7O_6PBa)_2 \cdot Ba(NO_3)_2$. The residue was washed twice with 6 cc of distilled water and several times with absolute ethyl alcohol and then ether. It was dried to a constant weight of 2,6441 g over phosphorus pentoxide under a high vacuum and had the following composition.

41,902 mg substance: 33,000 mg $BaSO_4$
4,120 " " : 20,525 " ammonium phosphomolybdate
8,906 " " : 0,256 cc N_2 (762 mm at 26,5°)
$(C_3H_7O_6PBa)_2 \cdot Ba(NO_3)_2$ Calculated. Ba 47,03, P 7,07, N 3,19
Found. " 46,34 " 7,23, " 3,28

Barium-β-glycerophosphate with one molecule of water of crystallization was present to the extent of 68,0 per cent of the theoretical and when the solubility of the double salt in the filtrate and wash water was considered (about 0,01 g per cc of water), the percentage reached 86,4.

The filtrate and water washings from the double-salt precipitation were treated with an excess of lead acetate solution and the insoluble lead glycerophosphate that formed was centrifuged out and washed free from soluble salt by centrifuging and decanting. After conversion to the barium salt the product was dried for 40 hours over phosphorus pentoxide at low vacuum and weighed 0,85 g.

It had the following composition and rotation. Sample dried at 138° at low vacuum over sulfuric acid.

7,700 mg substance: 3,395 mg CO_2 and 1,818 mg H_2O
4,005 " " : 30,200 " ammonium phosphomolybdate
11,505 " " : 8,015 " $BaSO_4$
$C_3H_7O_6PBa$. Calculated. C 11,71, H 2,28, P 10,10, Ba 44,69
Found. " 12,02, " 2,64, " 10,95, " 40,99

$$[\alpha]_D^{25} = \frac{-0{,}11° \times 100}{2 \times 16{,}67} = -0{,}33° \text{ (in water)}$$

1,97 g of the double salt were suspended in 20 cc of distilled water and 0,29 g of anhydrous sodium sulfate in 10 cc of water added. The amount of sodium sulfate used was about 10 per cent less than that required to precipitate the barium from the barium nitrate of the double salt. The suspension was shaken for one-half hour and centrifuged and the residue washed several times by centrifugation and decantation. The clear supernatant liquid and washings were treated with lead acetate solution and lead glycerophosphate precipitated. This was converted to the barium salt by the method used in a previous experiment.

The barium-β-glycerophosphate had the following composition. It was optically inactive.

Sample dried at 138° in a vacuum over sulfuric acid.

7,794 mg substance: 2,906 mg CO_2 and 1,680 mg H_2O
5,005 " " : 35,215 " ammonium phosphomolybdate
30,375 " " : 22,305 " $BaSO_4$
$C_3H_7O_6PBa$. Calculated. C 10,17, H 2,42, P 10,21, Ba 43,21
Found. " 11,71, " 2,28, " 10,10, " 44,69

Preparation of Tritosylglycerol. Two g of dry glycerol were dissolved in 5 cc of dry pyridine and a solution of 13,6 g of tosyl chloride (3,3 moles) in 15 cc. of pyridine slowly added while cooling the reaction mixture in an ice bath at 5°. Pyridine hydrochloride separated out almost immediately. The mixture was shaken at a temperature of 15° for two days and kept in an ice chest for 2 days more. After adding 1 cc of water to the brown mixture, it was cooled in ice, allowed to stand one-half hour and then diluted with 25 cc of water and extracted with chloroform several times. The combined chloroform extracts were washed successively with 5 per cent sulfuric acid, saturated sodium bicarbonate solution and finally water until neutral. It was then dried over anhydrous sodium sulfate, filtered and the filtrate evaporated under diminished pressure at 40° to a thick, almost colorless, sirup. 12 g were obtained. (Theoretical is 12,1 g).

After the addition of absolute alcohol and scratching with a glass rod, crystallization occurred. The white product was filtered and washed with absolute alcohol and ether. It was recrystallized once from absolute ethanol and twice from methanol when it melted at 101° (uncorr.) (Lit. 103°(5)).

The substance had the following composition.

4,002 mg substance: 7,595 mg CO_2 and 1,710 mg H_2O
5,312 " " 6,735 " $BaSO_4$
$C_{24}H_{26}O_9S_3$ Calculated. C 51,94, H 4,73, S 17,35
Found. " 51,75, " 4,78, " 17,42

Action of Sodium Iodide on Tritosyl Glycerol. 0,500 g of dry tritosylglycerol was dissolved in 5 cc of reagent acetone and 1 g of dry sodium iodide added. The mixture was heated in a sealed tube at 100° for 2 hours. The solution turned dark brown and a considerable amount of a crystalline substance separated out. The contents of the tube were filtered and washed free from iodine and sodium iodide with dry acetone and the insoluble residue dried to constant weight in a vacuum desiccator over phosphorus pentoxide. 0,502 g of sodium-p-toluenesulfonate was isolated.

$C_7H_7SO_3Na$. Calculated for the removal of 3 tosyl groups: 0,525 g
" " " " " 2 " " : 0,350 "

In another experiment 0,500 g of tritosylglycerol gave 0,470 mg of sodium p-toluene sulfonate.

The substance had the following composition:

13,000 mg substance: 4,856 mg Na_2SO_4

$C_7H_7SO_3Na$ Calculated. Na 11,85

Found. " 12,09

Preparation of Ditosyl α-Methylglycerol. A solution of 3 g of α-methyl ether of glycerol (6) in 15 cc of dry pyridine was cooled to 0° and 11 g (2,2 moles) of tosyl chloride added. Heat was generated and the solution was cooled to 0° and placed in a refrigerator for two days. The solution containing crystalline pyridine hydrochloride was worked up as described in the tosylation of glycerol. A light brown sirup was obtained which weighed 11 g (Theoretical yield, 11,7 g).

The sirup was purified by refluxing for one-half hour in methanol containing activated charcoal, filtering and distilling off the solvent in the filtrate at reduced pressure. The residual colorless sirup could not be crystallized and had the following composition.

4,022 mg substance: 7,710 mg CO_2 and 1,990 mg H_2O

5,000 " " : 5,691 " $BaSO_4$

$C_{18}H_{22}O_7S_2$ Calculated. C 52,13, H 5,35, S 15,47

Found. " 52,27, " 5,53, " 15,63

Action of Sodium Iodide on Ditosyl α-Methylglycerol. 0,558 g of dry ditosyl α-methyl glycerol was dissolved in 5 cc of dry acetone and 1 g of sodium iodide added. The sealed tube was heated at 100° for 2 hours during which time the solution turned brown and a crystalline substance formed. The residue was filtered off and washed free from iodine and sodium iodide with dry acetone. After drying to constant weight the sodium-p-toluene sulfonate residue weighed 0,486 g.

$C_7H_7O_3SNa$ Calculated for the removal of 2 tosyl groups: 0,522 g

" " " " " 1 " group: 0,261 "

The substance analyzed as follows:

6,320 mg substance: 2,307 mg Na_2SO_4

$C_7H_7O_3SNa$ Calculated. Na 11,85

Found. " 11,81

In a duplicate experiment 0,481 g of the tosylated ether gave 0,411 g of dry sodium p-toluene sulfonate.

$C_7H_7O_3SNa$. Calculated for the removal of 2 tosyl groups: 0,451 g

" " " " " 1 " group: 0,225 "

Oxidation of Barium-α-Glycerophosphate (3). 2 g of barium α-glycerophosphate (7) suspended in 15 cc of water were treated with 100 cc of bromine water (8) and allowed to stand at room temperature for 24 hours. The excess bromine was removed by aeration after the addition of 1 cc of concentrated sulfuric acid. 20 cc more of concentrated sulfuric acid were added and the colorless solution distilled.

To about 75 cc of the distillate an acetic acid solution of p-nitrophenylhydrazine was added in excess and the orange-red methylglyoxal p-nitrophenyl-osazone precipitated out slowly on heating to 80° for several minutes. After one crystallization from toluene the product decomposed with effervescence at 288° (uncorr.) [The literature gives 291—293° (9) uncorr.)]. Two more re-crystallizations from ethyl alcohol gave a melting point of 293° (uncorr.) (301° corr.). A mixed melting point with methylglyoxal p-nitrophenyl-osazone prepared from dihydroxyacetone was 290° (uncorr.).

The composition of the substance was the following:

1,115 mg substance: 0,238 cc N_2 (770 mm at 24,5°)

$C_{15}H_{14}N_6O_4$. Calculated. N 24,79
Found. " 24,75

Oxidation of Sodium-β-Glycerophosphate. 2,5 g of commercial sodium-β-glycerophosphate which had been recrystallized from water three times were dissolved in 100 cc of bromine water. After standing for 24 hours at 15° the reaction mixture was worked up as in the previous experiment.

Only a trace of orange-red precipitate was obtained after the addition of p-nitrophenylhydrazine in acetic acid solution indicating that practically no oxidation to a compound that could form methylglyoxal with sulfuric acid had occurred.

Oxidation of α-Methylglycerol (10). 2 g of α-methylglycerol were dissolved in 12 cc of water and 7 g of hydrated sodium carbonate were added. The solution was cooled to 10° and 3 g of bromine poured in. After standing for 30 minutes at room temperature the solution was acidified with hydrochloric acid and the excess bromine removed with sulfur dioxide.

The colorless solution was made neutral to Congo red with sodium hydroxide solution and a water solution of 1 g of p-nitrophenylhydrazine and 7 g of sodium acetate added. On warming, the orange-red p-nitrophenyl-osazone of α-methylglycerol formed. After two crystallizations from alcohol and one from toluene the compound decomposed with effervescence at 260—262° (uncorr.) 267—268° (corr.). The compound had the following composition:

3,590 mg substance: 0,696 cc N_2 (768,5 mm at 26°)

$C_{16}H_{16}N_6O_5$. Calculated. N 22,58
Found. " 22,37

Oxidation of β-Methylglycerol. 1 g of β-methylglycerol (11) was shaken with 75 cc of bromine-water for 15 minutes and allowed to stand at room temperature during 24 hours. The bromine was removed by aeration and the solution distilled with 15 cc of concentrated sulfuric acid.

The distillate gave only a trace of an orange precipitate with p-nitrophenylhydrazine on heating to 80°, indicating that practically no oxidation to a compound that could form methylglyoxal with sulfuric acid had occurred.

Oxidation of α,α'-Distearylglycerol. 3 g of distearin (12) were treated with 80 cc of bromine water and 20 cc of chloroform. The reaction mixture was shaken for 48 hours at room temperature. After removal of the bromine and chloroform, enough water was added to make 100 cc and 20 cc of concentrated sulfuric acid were carefully poured in.

The distillate from this mixture gave an osazone (with p-nitrophenylhydrazine) which melted with decomposition at 288—291°. Using a micro melting point apparatus with a polarizing microscope the original crystals were observed to undergo a transformation to fine needles at about 263° until melted. A mixed melting point with methylglyoxal p-nitrophenyl osazone was 286—288° (uncorr.). [291—293° (uncorr.) m.p. for this osazone of methylglyoxal].

Methylglyoxal p-nitrophenyl osazone is converted to fine needles beginning at about 258° until melted and this transformation appears to be characteristic of it. The phenomenon was not observed with the p-nitrophenylosazones of α-methylglycerol and glyceric aldehyde.

Oxidation of α,β-Distearylglycerol. 3 g of α,β-distearylglycerol (13) were dissolved in 20 cc of chloroform. 80 cc of bromine-water were added and the mixture shaken for 24 hours at room temperature. The bromine and chloroform were removed by aeration and 20 cc of water and 20 cc of concentrated sulfuric acid were added. The distillate gave an abundant orange-red precipitate with p-nitrophenylhydrazine.

Another experiment was performed with 2,5 g of the ester, 80 cc of bromine-water, 50 cc of phosphate buffer (ph = 7,3) and 20 cc of chloroform. This mixture was shaken for 16 hours at room temperature and after working it up in the usual way, the distillate gave an abundant orange-red precipitate on heating with p-nitrophenylhydrazine in acetic acid solution.

Oxidation of Lysolecithin. 2 g of lysolecithin were ground to an emulsion with 75 cc of bromine-water and then shaken for 24 hours at room temperature. The bromine was removed by an air current and the solution distilled after the addition of 15 cc of concentrated sulfuric acid.

The distillate yielded an orange-red osazone when heated to 80° with p-nitrophenylhydrazine in acetic acid solution. After recrystallization from glacial acetic acid and then ethyl alcohol, the compound melted at 293° (uncorr.) 301° (corr.).

It gave the needles characteristic of the p-nitrophenylosazone of methylglyoxal (about 260° until melted).

The substance had the following composition:

4,202 mg substance: 0,916 mg N_2 (745 mm at 24°)
3,495 " " : 6,720 " CO_2 and 1,285 mg H_2O.

$C_{15}H_{14}N_6O_4$.	Calculated.	N 24,79,	C 52,61,	H 4,12
	Found.	" 24,56,	" 52,43,	" 4,11

In a control oxidation experiment with choline, a possible fission product of lysolecithin, no methylglyoxal was obtained.

Attempted Methylation of Lysolecithin. 3 g of lysolecithin were dissolved in 5 cc of chloroform (dried and freshly distilled) and 10 cc of dry freshly-distilled methyl iodide were added. 2,5 g of silver oxide were added over a 2 hour period while the reaction mixture was being stirred and refluxed at 43—47°. After refluxing for 4 hours the mixture was allowed to stand overnight and was then extracted several times with chloroform. A viscous amber oil was obtained as the residue after evaporation of the chloroform at 40° under diminished pressure. The oil solidified at about 20°.

The product was remethylated in 5 cc of chloroform and 10 cc of methyliodide using 3 g of silver oxide. Another methylation in 20 cc of chloroform with 10 cc of methyl iodide and 3 g of silver oxide gave an oil which solidified at about 18°. A fourth methylation in 40 cc of dry benzene with 10 cc of methyl iodide and 3 g of silver oxide for 5 hours at 40—48° gave 2 g of an oil which solidified at 25°. After drying under high vacuum for 24 hours it had the following composition.

5,201 mg substance: 11,590 mg CO_2 and 4,912 mg H_2O
7,195 " " : 24,310 " ammonium phosphomolybdate
12,812 " " : 0,050 cc N_2 (762 mm at 29°)
Found C 60,57, H 10,57, P 4,90, N 0,45.
Calculated for the introduction of 2 methyl groups into lysolecithin
Containing stearic acid: C 58,99, H 10,62, P 5,44, N 2,45
" palmitic " : " 57,62, " 10,42, " 5,72, " 2,58

The low nitrogen and phosphorus analyses indicated decomposition.

The methylation product was dissolved in acetone and the solution cooled in ice water. A white wax settled out which was filtered at 12° and recrystallized from acetone. It had the following analysis.

3,105 mg substance: 7,983 mg CO_2 and 3,333 mg H_2O
1,816 " " : 3,280 " ammonium phosphomolybdate
7,311 " " : 0,035 cc N_2 (762 mm at 22°)
Found: C 70,01, H 12,01, P 2,61, N 0,55
Calculated for methyl stearate C 75,61, H 13,28
" " " palmitate " 75,47, " 12,67

From the filtrate of the compound described above another fraction was isolated at about —40°. This was filtered off quickly in a refrigerator at 0° and after the removal of adhering solvent under high vacuum a fluffy white substance remained.

The substance had the following composition:

4,120 mg substance: 10,310 mg CO_2 and 4,300 mg H_2O
6,270 " " : 10,112 " ammonium phosphomolybdate
No nitrogen was found.
Found: C 68,24, H 11,67, P 2,36, N 0,00

This fraction is probably a mixture of methyl stearate and palmitate with some methyl glycerophosphate.

The final filtrate was a mixture of decomposition products from which no identifiable products could be obtained.

Other methylation experiments using initially methanol, methyl iodide and silver oxide and also the combination chloroform, methyl iodide and silver carbonate yielded nothing but decomposition products and unreacted lysolecithin.

SUMMARY

The authors have tried to determine the position of the fatty acid in α-lysolecithin by three methods. Although the experiments failed

to elucidate the position of the fatty acid residue, they have shown some peculiar properties of α-lysolecithin which it has in common with other acylated glycerols.

1) Prepared by Dr. Ida Rolf.—2) P. Karrer, H. Salomon, Helv. Chim. Acta **9,** 7 (1926).—3) O. Bailly, Ann. de Chim. Physique, (9), **6,** 110 (1916).—4) P. A. Levene, R. S. Tipson, Jl. of Biol. Chem. **105,** 419 (1934).—5) A. Fairbourne, G. E. Foster, Jl. of Chem. Soc. **127,** 2759 (1925).—6) H. Hibbert, J. G. Morazain, Canad. Jl. Res., **2,** 215 (1930).—7) E. Fischer, E. Pfähler, Ber. Chem. Ges., **53,** 1615 (1920).—8) The bromine-water used in all of the oxidation experiments contained 3 cc of bromine in 1000 cc of solution.—9) P. A. Levene, A. Walti, Jl. of Biol. Chem. **68,** 420 (1926).—10) I. S. Neuberg, Bioch. Zs. **238,** 459 (1931).—11) H. S. Hill, M. S. Whelen, H. Hibbert, Jl. Amer. Chem. Soc. **50,** 2235 (1928).—12) A. Heiduschka, H. Schuster, Jl. für prakt. Chem. **120,** 149 (1929).—13) F. Guth, Zs. für Biol. **44,** 86 (1903).

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THE VERATRINE ALKALOIDS

II. FURTHER STUDY OF THE BASIC DEGRADATION PRODUCTS OF CEVINE

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(Received for publication, June 5, 1937)

Since our recent communication[1] on the degradation of cevine with zinc dust and soda lime, additional data have been obtained, which may bear some relationship to the nature of the dicyclic tertiary base, $C_{10}H_{19}N$, there described and may ultimately prove of significance in the interpretation of the structure of cevine itself.

In following further the products of the zinc dust distillation, it was found that the tertiary base $C_7H_{15}N$ was formed in larger amount than first suspected. This was made possible by a preliminary approximate separation of the basic mixture into strong and weak bases; *i.e.*, those of the saturated and those of the pyridine series, and finally by separation of the strong base fraction into tertiary and secondary base fractions with nitrous acid. It was further found that the base $C_7H_{15}N$ is optically active and was identified as *d*-N-methyl-β-pipecoline by direct comparison with its enantiomorph. This was prepared by cleavage of *dl*-β-pipecoline by the method of Ladenburg[2] and by conversion of pure *l*-β-pipecoline into the N-methyl derivative, according to the method used by Jacobi and Merling[3] for the racemic base. The mixture of the picrate of the resulting active base with the substance obtained from cevine proved indistinguishable in properties from the picrate of *dl*-N-methyl-β-pipecoline. This procedure was found necessary, since the racemic salt had a lower melting point than either of the active salts.

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[1] Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, **119**, 141 (1937).

[2] Ladenburg, A., *Ber. chem. Ges.*, **27**, 75 (1894); *Ann. Chem.*, **364**, 227 (1908).

[3] Jacobi, W., and Merling, G., *Ann. Chem.*, **278**, 6 (1893).

The secondary base fraction obtained by hydrolysis of the nitroso compounds gave an appreciable amount of β-pipecoline (apparently mostly racemic) which was isolated as the hydrochloride. Its identity was confirmed by the preparation of a *3,5-dinitrobenzoyl derivative* which was compared with that obtained from synthetic β-pipecoline. No evidence of the presence of coniine in this secondary base fraction could be obtained.[4]

In view of this experience we returned to a further study of the lower boiling fraction of the mixture of hydrogenated bases which resulted from the distillation of cevine with soda lime. After separation into secondary and tertiary basic fractions, an examination of the former showed it to be a mixture. In one experiment, partly active β-pipecoline (*d* base) was obtained in appreciable amount, and its nature confirmed by the preparation of the 3,5-dinitrobenzoyl derivative. However, in another experiment an extremely small amount, relatively, of a secondary base was obtained, which was converted into the 3,5-dinitrobenzoyl derivative. Analysis indicated it to be the acyl derivative of a propylpiperidine and this seemed to be confirmed by direct comparison with the acyl derivative prepared from *d*-coniine, although the rotation indicated extensive racemization. This result may thus be regarded as partial confirmation of the earlier report of Macbeth and Robinson[4] who described, however, the isolation of the antipode *l*-coniine from cevine. However, when opportunity is found, this point will be investigated further. Unfortunately, in our hands it has seemed essential to sacrifice a very large amount of cevine for the purpose. Our experience has shown that the reaction distillate is much more complex than would appear from the report of Macbeth and Robinson, and the detection of coniine more difficult.

In the weak base fraction we have already reported the isolation of a base $C_8H_{11}N$ as a picrate which melted at 132–133°. The attempt has since been made to determine its identity by oxidation, since its properties did not correspond with those of any recorded substance. On oxidation with permanganate a dibasic acid was obtained, which melted at 270–272° with decomposition and was readily decarboxylated to nicotinic acid. This ready decarboxylation indicated an α-carboxyl group and together with recorded data on the pyridine dibasic acids pointed definitely to pyridine-2,5-dicarboxylic acid or isocinchomeronic acid. The formation of such a dibasic acid would indicate, therefore, that the base, $C_8H_{11}N$, is either 2-ethyl-5-methyl pyridine or 2-methyl-5-ethyl pyridine. However, the picrate of the latter has been described[6] as melting at 164°, thus leaving the former substance as the probable one. When opportunity presents, an attempt will be made to prepare this base synthetically for comparison with the cevine degradation product.

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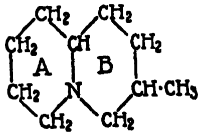
[4] Macbeth, A. K., and Robinson, R., *J. Chem. Soc.*, **121**, 1571 (1922).

A preliminary attempt can be made to interpret these observations. If the previously described dicyclic base $C_{10}H_{19}N$, which was obtained on soda lime distillation with subsequent hydrogenation, is given the formula of a methyl octahydropyridocoline (or of a dimethyl octahydropyrrocoline)



it is possible that an unsaturated precursor possessing such a ring system and formed from cevine on zinc dust distillation could give an N-methyl-β-pipecoline as well as β-pipecoline itself by cleavage of Ring A. Similarly, a pyridine base by dehydrogenation and cleavage of Ring A could give a 2-ethyl-5-methyl pyridine. Finally, the formation of β-pipecoline and possibly α-propylpiperidine (coniine) or a homologue might be similarly explained. Whether a dicyclic base of this type occurs as such in cevine and is attached by a side chain or directly fused to the remainder of the molecule can be answered only by further work.

Further investigation of the non-basic portion of the cevine molecule is now in progress.

EXPERIMENTAL

Zinc Dust Distillation of Cevine—This operation was performed essentially as previously described[1] with the exception that a rough separation of weak and strong bases was made as follows. In an experiment starting from 60 gm. of cevine about 80 cc. of additional acetic acid were added to the solution after hydrogenation and the mixture was steam-distilled into dilute hydrochloric acid. After collection of about 2 liters of distillate which carried over a mixture of weak bases and non-basic material, the residue was made alkaline with sodium hydroxide and the steam distillation resumed. The volatile, strong base fraction was collected in dilute hydrochloric acid. After concentration of the resulting solution to a syrup, the strong bases were liberated with potassium hydroxide and extracted with about 40 cc. of ether. The resulting extract was dried over KOH and then fractionated.

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[6] Fichter, F., and Labhardt, H. P., *Ber. chem. Ges.*, **42**, 4714 (1909).

The first fraction which consisted mainly of ether was condensed in a receiver chilled with ice and connected also with a dry ice trap. On addition of ethereal picric acid to the distillate and rinsings from the trap, copious crystallization occurred of the picrate which melted at 165–170°. The yield was 0.135 gm. and analysis showed it to be the salt of the base $C_7H_{15}N$.

$C_7H_{15}N \cdot C_6H_3O_7N_3$. Calculated, C 45.59, H 5.30; found, C 45.90, H 5.26

After transferral of the undistilled oil to a microstill, a rough fractionation was resumed and material was collected which distilled up to 145° at 760 mm. This fraction after dilution with ether yielded a picrate which was the salt essentially of the base $C_7H_{15}N$. After recrystallization from alcohol, 0.33 gm. of stout needles was obtained, which melted at 171–176°. 80 mg. of less pure picrate were recovered from the mother liquor. Finally, when the above distillation was carried further, it was found that the higher boiling material gave only inappreciable amounts of crystalline picrate. The total yield of crude picrate was roughly 0.57 gm.

After repeated recrystallization from alcohol a micromelting point of 176–178° was obtained.

$[\alpha]^{23}_{5780} = +9.7°$ ($c = 1.02$ in acetone)

$C_7H_{15}N \cdot C_6H_3O_7N_3$. Calculated. C 45.59, H 5.30, CH_3 4.39
Found. " 45.80, " 5.01, " 3.43

dl-N-Methyl-β-pipecoline was prepared according to Jacobi and Merling[3] by distillation of N-dimethyl-β-pipecolinium chloride. The crude base gave a picrate which after recrystallization from alcohol formed crystals which appeared broader than those from the cevine base and also had a lower melting point, *viz.* 165–168°. Found, C 45.95, H 5.20, CH_3 3.40.

However, since this synthetic material was racemic, an active β-pipecoline was first prepared according to Ladenburg[3] by cleavage with *d*-tartaric acid. *l*-Pipecoline-*d*-tartrate was thus obtained, which melted at 173–175°.

l-N-Dimethyl-β-pipecolinium iodide was prepared from *l*-β-pipecoline in methyl alcoholic solution with methyl iodide and sodium hydroxide. It formed prismatic needles from alcohol, which melted at 200–201° and showed $[\alpha]_D^{28} = +7.0°$ ($c = 1.0$ in water).

$C_8H_{18}NI$. Calculated, C 37.64, H 7.11; found, C 38.08, H 7.16

The chloride prepared from this salt with silver chloride was converted by distillation into *l*-N-methyl-β-pipecoline which was in turn isolated as the picrate. The latter after recrystallization from alcohol formed stout needles indistinguishable in appearance from the picrate obtained from cevine. It melted at 176–179°. However, $[\alpha]_{5780}^{23} = -12.6°$ ($c = 1.03$ in acetone). Found, C 45.77, H 5.44. Since the rotation of the picrate from cevine indicated that it contained a small amount of the racemic form, a mixture of 30 mg. of this picrate and 25 mg. of the above synthetic *l* derivative was recrystallized from alcohol. 47 mg. of a mixture were recovered, which appeared indistinguishable in form from the picrate of synthetic *dl*-N-methyl-β-pipecoline. Like the latter it melted at 164–168°, and, when mixed with it, showed no depression.

The free base recovered from the picrate from cevine was converted, for further characterization, into the quaternary salt with methyl iodide in ether solution. After recrystallization the resulting *d*-N-dimethyl-β-pipecolinium iodide melted at 199–201°.

$C_8H_{18}NI$. Calculated, C 37.64, H 7.11; found, C 37.70, H 6.72

At a subsequent stage of the work and following preliminary studies, further separation of the strong base fraction into secondary and tertiary bases was carried out over the nitroso compounds as follows.

Material was used which had been collected from a number of experiments, representing a total of 225 gm. of cevine which had been distilled with zinc dust. This included the mother liquors from the experiment described above, which gave 0.57 gm. of crude picrate of N-methyl-β-pipecoline. The mixture of strong bases in all of this material was again distilled with steam into dilute hydrochloric acid and the resulting solution was concentrated *in vacuo* to a syrup. This syrup of hydrochloride was dissolved in 10 cc. of water and treated with an excess of 30 per cent sodium nitrite solution. Oily nitroso compounds gradually separated. After standing at room temperature for 24 hours, the appreciable oily layer was extracted with a small volume of ether.

The aqueous fraction containing tertiary bases was made alkaline and distilled with steam into dilute hydrochloric acid. Concentration of the latter *in vacuo* left a partly crystalline residue of the hydrochloride. In a preliminary experiment a hydrochloride was obtained as needles from alcohol-ether, which melted at 180–192°. The analysis indicated it to be the salt of N-methyl-β-pipecoline.

$C_7H_{15}N \cdot HCl$. Calculated, C 56.15, H 10.78; found, C 55.44, H 10.70

However, because of its hygroscopic character the main material was converted into the picrate. After repeated recrystallization from alcohol, 0.95 gm. of picrate was obtained, which melted after preliminary softening at 165–171°.

$C_7H_{15}N \cdot C_6H_3O_7N_3$. Calculated, C 45.59, H 5.30; found, C 46.24, H 5.39

The ether solution of crude nitroso derivatives (described above) was mixed with an equal volume of alcohol and then an equal volume of concentrated hydrochloric acid. This mixture after standing at room temperature overnight was refluxed several hours and then concentrated to a syrup. This was desiccated by repeated concentration with absolute alcohol. The partly crystalline residue was finally dissolved in a small volume of absolute alcohol and on careful addition of dry ether yielded an oil followed by gradual crystallization which was increased by further addition of ether. The crystals were pipetted from persistent oily material and collected with ether. 0.85 gm. was obtained.

After recrystallization from alcohol-ether the salt formed needles which melted at 171°. No rotation could be detected in 1 per cent aqueous solution. Analysis showed it to be a pipecoline.

$C_6H_{13}N \cdot HCl$. Calculated. C 53.10, H 10.41, N 10.33
Found. " 53.17, " 10.46, " 10.30

For confirmation of its identity the base was converted into the 3,5-dinitrobenzoyl derivative by acylation in benzene solution with the acid chloride and dilute sodium hydroxide solution. The acyl derivative separated from 95 per cent alcohol as leaflets which melted at 111–113° after preliminary sintering.

$C_{13}H_{15}O_5N_3$. Calculated, C 53.22, H 5.16; found, C 53.17, H 5.04

This material showed no depression when mixed with 3,5-dinitrobenzoyl-β-pipecoline which melted at the same point and which was prepared from *dl*-β-pipecoline. The latter was obtained by hydrogenation of β-picoline which in turn had been prepared according to Stoehr[6] but finally purified as the picrate. The acyl compound gave the following analytical figures. Found, C 53.37, H 5.14. Attempts to identify any other base, and especially coniine, in the secondary base fraction have thus far been unsuccessful.

Soda Lime Distillation

In experiments involving distillation of 110 gm. of cevine with soda lime, as previously described, the fraction of bases boiling up to about 175° at 760 mm. as well as basic material recovered from the mother liquor of the picrate, $C_{10}H_{19}N \cdot C_6H_3O_7N_3$, were combined and finally obtained as a syrup of hydrochlorides. This material in concentrated solution was treated with 30 per cent sodium nitrite. Oily nitroso compounds gradually separated. After several hours the oil was extracted with a small volume of ether and hydrolyzed. After it was made alkaline, the secondary bases were steam-distilled into dilute hydrochloric acid and concentrated to dryness. In this case, dry ether added to the alcoholic solution precipitated first resinous material which was removed with pure bone-black. On further addition of ether to the filtrate and long standing, slow crystallization occurred. This was collected with ether. In view of the extremely small yield, about 50 mg., this material was acylated for characterization with 3,5-dinitrobenzoyl chloride. The reaction product was finally obtained as an oil which was treated with a drop or so of alcohol. All attempts to seed it with the *β*-pipecoline derivative were unsuccessful. However, the derivative of *d*-coniine caused prompt crystallization. After recrystallization from alcohol it slowly melted at 100–107° after preliminary sintering. It gave a strong depression with dinitrobenzoyl-*β*-pipecoline but not with dinitrobenzoyl-*d*-coniine.

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[6] Stoehr, *J. prakt. Chem.*, **43**, 154 (1890).

$[\alpha]_D^{28} = +11.7°$ ($c = 0.515$ in acetone)
$C_{15}H_{19}O_5N_3$. Calculated, C 56.04, H 5.96; found, C 55.87, H 5.62

The rotation of our previously described dinitrobenzoyl-*d*-coniine has since been found to be $[\alpha]_D^{25} = +49°$ ($c = 1.025$ in acetone).

In an attempt to confirm this result a similar fraction of bases resulting from the distillation of 70 gm. of cevine was similarly investigated.

In this case the concentrated solution of hydrochlorides of recovered bases was allowed to stand 24 hours after addition of sodium nitrite solution. The appreciable oily material which formed was extracted with ether and hydrolyzed. In this experiment a larger crystalline fraction was obtained than in the former case. 0.17 gm. of hydrochloride was collected from alcohol-ether and melted at 168–171° upon recrystallization.

$C_6H_{13}N \cdot HCl$. Calculated, C 53.10, H 10.41; found, C 52.94, H 10.48

The substance thus appeared to be the salt of *β*-pipecoline.

For further characterization the 3,5-dinitrobenzoyl derivative was made, which melted at 110–113°. It proved, however, to be optically active.

$[\alpha]_D^{30} = +8°$ ($c = 0.5$ in acetone)
$C_{13}H_{15}O_5N_3$. Calculated, C 53.22, H 5.16; found, C 53.20, H 5.06

In order to ascertain the optical character of this material the 3,5-dinitrobenzoyl derivative was prepared from *l*-*β*-pipecoline. It formed needles or leaflets which melted at 114–116° after preliminary sintering. $[\alpha]_D^{32} = -30°$ ($c = 1.00$ in acetone). Found, C 53.33, H 5.24.

Thus the rotation indicated that the above substance from cevine was the derivative of largely racemized *d*-*β*-pipecoline.

Further investigation of material contained in the mother liquors from this hydrochloride failed to give any certain indication of the presence of coniine. A small amount of crystalline material was obtained as a hydrochloride which proved to be an obvious mixture. Its analysis and the small amount of crystalline acyl derivative which could be obtained from it gave indications of a possible mixture of pipecoline and coniine or even of a dimethyl piperidine.

Oxidation of the Base $C_8H_{11}N$

0.2 gm. of the previously described picrate melting at 132°[1] was treated with 5 cc. of 10 per cent hydrochloric acid and picric acid was extracted with ether. The acid solution was evaporated to dryness *in vacuo* and the residue dissolved in a few drops of water. It was made neutral to litmus with potassium carbonate solution and treated with 0.6 gm. of potassium permanganate. The volume was then diluted to 30 cc. and the mixture was heated at 100° for 6 hours. The excess permanganate was removed with a slight excess of sodium bisulfite. The clear filtrate from manganese dioxide was exactly neutralized to Congo red with hydrochloric acid, then treated with 0.53 cc. of N hydrochloric acid, and finally evaporated to dryness. The residue was dissolved in 4 cc. of water. On chilling, 55 mg. of crystalline material slowly separated, which melted at 270–272° with vigorous decomposition when heated rapidly. Further recrystallization did not raise the melting point.

$C_7H_5O_4N$. Calculated, C 50.30, H 2.99; found, C 50.49, H 2.82

Decarboxylation of the Dibasic Acid—15 mg. of the acid were placed in a microstill[7] and heated under 25 mm. of pressure at an oil bath temperature of 270° until nothing more passed over. 11 mg. of crystalline material were removed mechanically from the condenser. This substance began to melt at 190–200°, but did not entirely melt until a temperature of 240° was reached. The distillate was again placed in the distillation apparatus and all material distilling up to an oil bath temperature of 150° and under 0.25 mm. of pressure was collected. It melted at 223–226°. The higher boiling material remaining in the residue gave more of the lower melting material upon being distilled again at the higher temperature as above. The material melting at 223–226° on redistillation at 150° and 0.25 mm. of pressure gave a product melting at 225–227°.

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[7] Craig, L. C., *Ind. and Eng. Chem., Anal. Ed.*, 8, 219 (1936).

$C_6H_5O_2N$. Calculated, C 58.54, H 4.06; found, C 58.82, H 3.98

The three pyridine monocarbonic acids melt as follows: picolinic acid at 135°, nicotinic at 229–230°, and isonicotinic at 309°. Of the six possible pyridine dicarbonic acids, that substituted in the 3,5 positions melts at 322°,[8] and that in the 2,3 positions at 180° with decomposition.[9] The isomer substituted in the 2,4 positions could give only picolinic acid or isonicotinic acid upon decarboxylation, while that substituted in the 2,6 positions could give only picolinic acid. The acid from cevine described above must therefore be either the 2,5 or the 3,4 isomer. The 3,4 isomer gives upon decarboxylation a mixture of nicotinic and isonicotinic acids.[10] In the above decarboxylation experiment, however, no monocarboxy acid could be isolated which melted above 227°. Furthermore, the 3,4 derivative yields readily an anhydride when heated with acetic anhydride according to the directions of Strache.[11] Our acid gave only unchanged dibasic acid when subjected to this treatment. By a process of elimination the acid from cevine must therefore be the 2,5-pyridine dicarbonic acid or isocinchomeronic acid. Several values are given in the literature for its melting point, an uncertainty probably caused by its decomposition below the melting point to give nicotinic acid.[12]

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[8] Guthzeit, M., and Dressel, O., *Ann. Chem.*, **262**, 131 (1890).

[9] Hoogewerff, S., and van Dorp, W. A., *Ann. Chem.*, **204**, 117 (1880).

[10] Hoogewerff, S., and van Dorp, W. A., *Ann. Chem.*, **204**, 113 (1880).

[11] Strache, H., *Monatsh. Chem.*, **11**, 134 (1890).

[12] Weidel, H., and Herzig, J., *Monatsh. Chem.*, **1**, 16 (1879); **6**, 982 (1885).

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A FRACTIONAL-DISTILLATION MICROAPPARATUS

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(Received for publication, May 7, 1937)

The development of organic micromanipulation has been largely due to the necessity of using it in the study of rare natural products or very costly materials. It has certain advantages, such as speed, economy, etc., and also has limitations. A very serious limitation, as compared with the customary organic macroprocedures, is that of fractional distillation. Although several apparatus for microdistillation and in a degree for microfractionation (*1*, *2*) have been reported, no attempt has been made to reach the precision attained in macrowork by the use of carefully controlled packed columns, with the exception of Podbielniak's still (*4*) which requires a few cubic centimeters or more.

Since in the degradative study of certain natural products, the author has constantly been confronted with the need of more precise fractional distillation of small quantities, he has attempted to develop a more efficient apparatus. Results of the study at this point seem to justify a preliminary communication, although the work is still in progress.

Design of Apparatus

Although the principle is the same as that employed in macrocolumns, the requirements for proper execution are somewhat different. Obviously an extremely small holdup becomes necessary. This requirement, however, can be partially counterbalanced by the fact that the capacity of the microcolumn can be extremely small and proportionately small reflux is thus required. Bearing in mind these factors, the apparatus shown in Figure 1, designed for volumes of 0.5 to 2.0 gram of material, has been devised. When approximately 0.3 gram of distillate was collected per hour, it gave a separation of benzene–carbon tetrachloride mixture corresponding to approximately 8 theoretical plates according to the method of Fenske and co-workers (*3*). It has a holdup (weight of material in apparatus when distilling flask has just become dry) of approximately 0.1 gram for

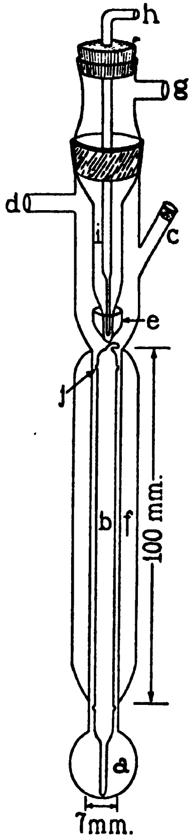

FIG. 1

these liquids, the column being 10 cm. in length and approximately 7 mm. in diameter. The apparatus (Figure 1) is constructed as follows:

The distilling flask, *a*, is a round bulb of 4-cc. capacity. Into the flask is sealed a tube which is as straight as possible but very thin-walled, made by drawing out from a large tube so that it is about 7 mm. in diameter. Experiments with the standard Pyrex tubing, in which the walls are 1 to 1.5 mm. thick, have given poor results. A good explanation of this seems to be lacking and must be investigated further. This tube, which serves as the outside of the column, is surrounded by a sealed-on glass jacket, *f*, for protection and insulation. The effect of silvering and evacuating the jacket has not yet been studied. A tube 15 cm. wide and 50 mm. long is sealed to the upper end of the column and has a ground-glass joint at its upper end, through which extends the condenser, *i*. This tube has an outlet tube, *d*, for evacuating when reduced pressure is used, and another outlet tube, *c*, fitted with a small ground-glass stopper.

The condenser, *i*, carries a rubber stopper at its top, through which extends a glass tube, *h*, for the entrance of water into the condenser, the water leaving the condenser through the exit tube, *g*. The lower end of the condenser tube is drawn out into a rather narrow thin tip which has sealed on its end a cup, *e*, which holds approximately 0.2 cc. Liquid condensing on the upper part of the condenser will run down and be caught in the cup, where it can easily be withdrawn through tube *c*, when a fraction is desired, by means of a small pipet. The inside part of the column is represented by *b*, which is a very thin-walled tube, sealed at both ends, of shape represented. It is not attached to the rest of the apparatus and can be removed at will by a small glass hook at its top. Its lower, drawn-out end rests on the bottom of flask *a* and the upper part is held away from the outside part of the column at a distance of about 1.5 mm. by six tiny glass nipples, 1.5 mm. long, which are sealed on the surface of tube *b*. The outside diameter of *b* must therefore be approximately 4 mm. in order to give the necessary 1.5-mm. clearance all around.

Heat is supplied to flask *a* by means of a small oil bath, which is kept constantly stirred mechanically with a small air-driven motor. A thermometer in the oil bath is the only measure of temperature possible for low-boiling liquids with this apparatus. Experiments with a tiny thermocouple at the top of the column were not promising.

The liquid to be fractionated is inserted with a pipet through the top of the apparatus before the inside part of column *b* is put in place. The temperature of the oil bath is then slowly raised until the ring of condensing liquid at the top of the vapor just reaches the tip of cup *e* or the top of the column. To do this, it is necessary to adjust the temperature of the oil bath to ±0.5°. Material will then slowly diffuse from the ring of liquid through the vapor phase up to the upper part of condenser *i* and then run down to cup *e*. In this way approximately 0.3 gram of liquid can be obtained in an hour, and this has been found by experiment to be about the capacity of the still for maximum fractionation with carbon tetrachloride–benzene mixtures. When run more quickly than this, the efficiency of the still was rapidly affected.

When operated in the way described, the inside part of the column gets its reflux from material condensing on the outside of cup *e* and a thin film of liquid accordingly runs down over the entire surface of *b*. In the corresponding way the outside part of the column gets its reflux from the top, this being exposed to the air just above jacket *f*. A uniform flow of liquid is thus furnished to the entire inside surface of the outside part of the column.

In fractionating a mixture, arbitrary fractions are withdrawn by means of a capillary pipet through *e*, and the boiling point, refractive index, etc., are taken on each fraction by well-known micromethods. For maximum operation there must be a small, constant temperature gradient between the inside part of the column and the immediate outside atmosphere. This gradient would be too large for high-boiling liquids, too much condensation would occur at the top of this column, and flooding would result. Too large a vapor velocity would be necessary to supply the reflux and the efficiency of the column would drop rapidly.

To overcome these difficulties, the apparatus is placed in a glass tube of approximately 3.5-cm. inside diameter which reaches from a point at the base of *c* to the lower part of jacket *f*, as shown in Figure 2. The glass tube is held in place on the apparatus by means of thin cork, 1, cut accordingly. The outside of the glass tube is wrapped with a suitable amount of Nichrome wire, *m*, so as to serve as a heating unit and is attached to a variable resistance. A thermometer, *n*, passes through the cork at the top of the glass tube and is thus suspended between jacket *f* and the glass tube. In this way the still can easily be maintained at a temperature best suited for its maximum performance

Tests on Synthetic Mixtures

In order to be sure of the value of a fractionating column, actual tests on the separation of synthetic mixtures are necessary. Figure 3 represents graphically some of the many tests made. The first mixture (mixture I) was a 50 per cent by weight mixture of chlorobenzene and toluene. These two liquids boil 21° apart. An ordinary distilling flask gave the result represented by curve 1 (Figure 3). Curve 2 is the separation given by a Vigreux column 18 cm. in length. Curve 4 is the separation given by the distillation apparatus described and illustrated in Figure 1 (10-cm. column), and curve 3 is the separation given by this column when the center stick (*b*, Figure 1) is removed from this column. All analyses given in this paper were made by the refractive index method and all the results are easily reproducible. Check runs on all the separations have been made.

In order to give a complete picture of a separation, the data of Table I are presented. For this distillation 1 gram of a 50 per cent mixture by weight of carbon tetrachloride–benzene (mixture II) was used. Amounts are given by weight rather than the customary mole fraction because the organic chemist is more interested in the relative weights. The separation was made in a still as described, except that the column was 22 cm. in length.

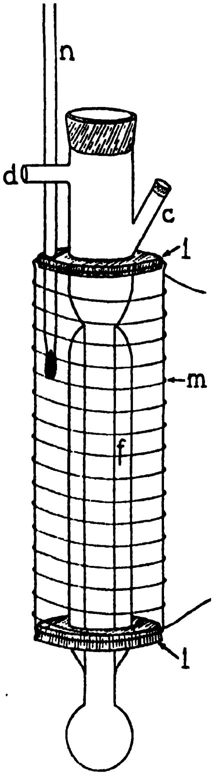

FIG. 2

These data, when represented graphically, give curve 5, Figure 3.

A study of the literature concerning macrofractionating columns reveals the fact that efficiency curves are always given for relatively small molecules and at atmospheric pressures. Most substances encountered by the organic chemist in laboratory work are larger molecules where it is necessary to use reduced pressure. With this in mind, a study of the behavior of this type of column under greatly reduced pressure is under way. Part of the data will be presented here.

The first mixture studied was methyl laurate–diphenyl ether (mixture III). With the column as described (22 cm. in length), very poor separations were obtained and the liquid did not appear to wet the glass surfaces of the column but collected in globules. When, however, the walls of the column and of the outside surface of the inside column were lined with a coil of No. 35 Nichrome wire similar to the column of Podbielniak (*4*), approximately 6 turns per cm., a uniform film of descending liquid was spread over the surface and curve 1, Figure 4, was obtained when the distillation was done under 0.20-mm. pressure and 1 gram of the mixture was used. An ordinary distilling flask gave with this mixture and under this pressure a distillate containing 67 per cent of the more volatile. Without the center part of the column (*b*, Figure 1), curve 2 was obtained, thus again con-

TABLE I

Distillation Data

Fraction	Weight	Time	Temperature of Oil Bath	Temperature of Column Jacket	Refractive Index	Per Cent More Volatile
	Gram	*Min.*	*°C.*	*°C.*		
1	0.070	14	86–7	42.5	1.4678	84.5
2	0.080	10	86.5–87.5	42.5	1.4678	84.5
3	0.100	12	87–87.5	42.5	1.4680	84.0
4	0.060	15	86.5–7	42.0	1.4676	85.0
5	0.050	14	87.5–8	42.0	1.4681	84.0
6	0.075	10	89–90	43.0	1.4706	80.0
7	0.055	12	90	43.0	1.4726	76.0
8	0.055	12	90–3	43.5	1.4767	67.5
9	0.060	7	93	44.0	1.4851	48.0
10	0.050	8	93–93.5	44.0	1.4910	31.0
11	0.050	10	93.5–94	44.0	1.4937	22.0
12	0.070	9	94–6	44.0	1.4962	10.5
13	0.050	10	96–8	44.0	1.4968	7.5
14	0.150	Residue in still		....	1.4974	5.0
	0.975	143				

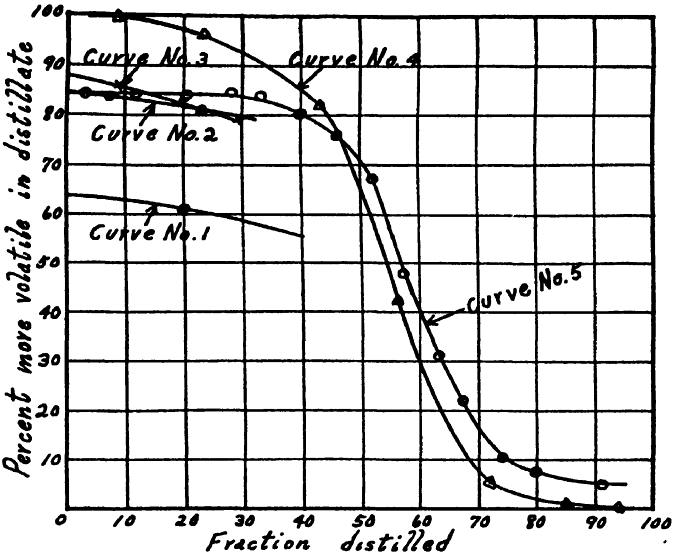

FIG. 3. Tests on Synthetic Mixtures
1, 2, 3, 4. Mixture I
5. Mixture II

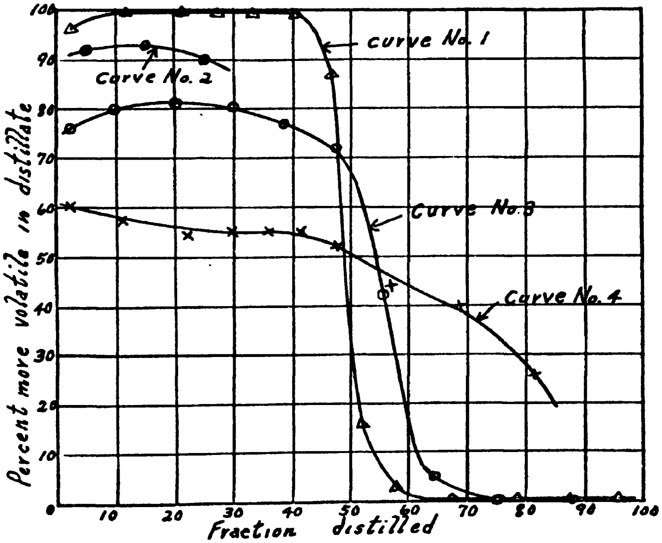

FIG. 4. Tests on Synthetic Mixtures
1, 2. Mixture III
3, 4. Mixture IV

firming the desirability of the center part (*b*, Figure 1) of the column. The results obtained with this mixture were duplicated by the results obtained with a 50 per cent mixture of α-chloronaphthalene and methyl laurate.

Curve 3, Figure 4, was obtained by distilling 1 gram of a mixture of 50 per cent α-chloronaphthalene and diphenyl ether (mixture IV) under 0.2-mm. pressure. This mixture, when distilled from a distilling flask under 0.2-mm. pressure, gave a distillate containing 52 per cent of the more volatile and the two components appear to form a constant-boiling mixture similar to carbon tetrachloride–benzene at approximately 95 per cent of the more volatile, as the column refused to give further separation when a mixture of this composition was distilled. With this curve and with the curve for mixture II the more efficient separation was obtained with the last part of the distillation—the bottoms.

Curve 4 represents the separation obtained when mixture IV was fractionated at 60-mm. pressure. An ordinary distilling flask gave, under 60-mm. pressure, a distillate containing not more than 50.3 per cent of the more volatile.

LITERATURE CITED

(1) Craig, L. C., Ind. Eng. Chem., Anal. Ed., **8**, 219 (1936).
(2) Emich-Schneider, "Microchemical Laboratory Manual," 2nd ed., New York, John Wiley & Sons, 1932.
(3) Fenske, M. R., Quiggle, D., and Tongberg, C. O., Ind. Eng. Chem., **24**, 408 (1932).
(4) Podbielniak, W. J., *Ibid.*, Anal. Ed., **5**, 119 (1933).

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ACTION OF VARIOUS DYESTUFFS ON FERMENTATION AND PHOSPHATE SYNTHESIS IN YEAST EXTRACT

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(*From the Laboratories of The Rockefeller Institute for Medical Research*)

(June 15, 1937)

In a previous paper from this laboratory (1) a study of the effect of a series of dyes on the aerobic fermentation of glucose by a yeast extract was reported. Three of the dyes of this series, naphthol sulfonate indophenol, brilliant alizarin blue and rosindulin G G, were found to inhibit the fermentation of glucose or of hexosemonophosphate, but not the fermentation of hexosediphosphate.

If diphosphate was added to an extract containing dye and glucose, a rapid production of CO_2 occurred until two molecules of CO_2 per molecule of added diphosphate had been formed. This fermentation not only produced no inorganic phosphate, but brought about a synthesis of organic phosphate from the inorganic originally present. The amount of organic phosphate synthesized was just equivalent to the amount of phosphate added as diphosphate. The reaction taking place could be represented by the following equation

$$C_6H_{10}O_4(PO_4M_2)_2 + 2M_2HPO_4 + 4ROH \rightarrow 2CH_3CH_2OH + 2CO_2 + 4RPO_4M_2 + 2H_2O$$

This equation holds particularly well in the case of rosindulin G G because the action of this dye is restricted to the process just mentioned. The other two dyes have essentially the same effect but, in addition, they increase the oxygen consumption of the extract, an effect which they share with many other reversibly reducible and oxidizable dyes. This latter effect is not exhibited by rosindulin G G, a property which greatly simplifies the study of the specific effect mentioned. The RPO_4M_2 formed under the influence of this dye has been isolated and found to be chiefly hexosemonophosphate (2). The utilization of this reaction has been recommended as a method of preparing hexosemonophosphate (2).

As far as the writers are aware the utilization of such dyes is the only method known by which a complete yeast extract can be made to act upon glucose plus hexosediphosphate and to stop at the monophosphate stage. Such a method should then be able to give us some information about the formation and the further utilization of this monophosphate.

For example, the equations given by Meyerhof and Kiessling (3) offer a ready explanation for the transference of the phosphate of the added diphosphate to glucose, to form monophosphate—but as far as we can see they offer no explanation of how an equivalent amount of inorganic phosphate is transferred to glucose. It is, however, not the purpose of the present paper to deal with such problems, but rather to present briefly the results of tests of a number of other dyes in an effort to determine what factors are necessary in a dye in order that it may show the above effect. By the above effect we understand the ability to repress the fermentation of glucose or monophosphate, but to permit the fermentation of diphosphate with the accompanying synthesis of a difficultly hydrolyzable ester.

It was stated in the previous paper that this effect of a dye on fermentation was not determined by its oxidation-reduction potential nor by the amount of oxygen consumption that it brought about. The present work has amply confirmed this. The most apparent factor upon which the effect might depend, is the chemical constitution of the dye.

The three dyes mentioned above have in common a naphthol ring and a sulphonate group, the latter, however, is not always located at the naphthalene ring. It was thought that either or both of these groups might be the effective factor. That substituted naphthol sulfonates have an effect on such processes as blood coagulation has been pointed out before (4). This effect was found to be increased about tenfold by the introduction of an azo group. As may be seen below, the naphthol sulfonate group alone is not sufficient to produce the effect in which we are interested, but almost every nitrogen-containing dye tested which contains these groups does produce the effect. However, a dye which contains neither a naphthol ring nor a sulfonate group may also produce the effect. Further, it should be emphasized that rosindulin G G, mentioned above as one of the most, if not the most efficient dye, contains the sulphonate group not at a naphthalene ring, but at a benzene ring. According to the information given in the color index rosindulin G, differs from G G only by having the sulphonic group at the naphthalene ring but it has no appreciable inhibiting effect on the fermentation of glucose.

It should also be emphasized that all these results were obtained with cell-free extract. The fermentation of living yeast is practically uninfluenced by any of the dyes mentioned, either aerobically or anaerobically. This is probably due to the fact that sulphonated dyes do not penetrate into the living cell.

PROCEDURE AND RESULTS

Some of the dyes used were procured from the sources given in Rowe's color index through the American agencies. The rest of the dyes and other compounds used were prepared in our laboratory.

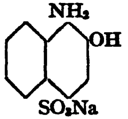
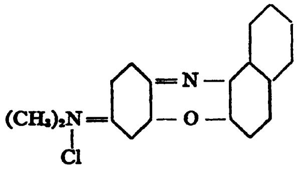
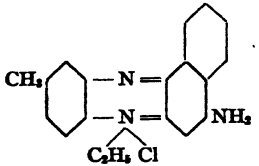
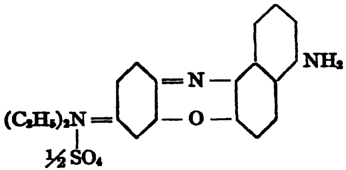
A list of the dyes tested and certain data concerning them is given in Table I. The dyes containing sulfonic acid side-chains are all sodium salts, except No. 7, and likely were all obtained by a final salting out procedure. Analyses on some samples showed that they are somewhat low in nitrogen, probably due to a certain content of NaCl from the mother liquor. The concentrations as tabulated are calculated from the weight of dye used and the molecular weight, so they may, in some cases, be not quite correct but the error is not a great one. The dyestuffs prepared in this laboratory are, according to analysis, pure preparations. We determined for each dye the optimum concentration for the effect desired. Since these values are not very sharp, a small error in the true concentration is of no particular interest. Certainly, even allowing for such an error in concentration, the optimum concentration for the effect desired varies considerably from dye to dye.

The procedure followed was to add the dye to be tested, in the concentration desired, to a preparation of „Lebedev-saft" having a final dilution of two-fold, and measuring the rate of CO_2 production manometrically in a Barcroft-Warburg apparatus after adding glucose and a small amount of hexosediphosphate. With the effective dyes, the CO_2 production stops when the diphosphate added has been utilized. More diphosphate is then added and the CO_2 produced is measured until it again ceases. The contents of the vessel are then deproteinized and analyzed directly for inorganic phosphorus, and for inorganic phosphorus formed after heating for 15 and 60 minutes at 100°C in 1,0 NH_2SO_4. The Fiske-Subbarow method was used for phosphorus determination. Colorimetry was carried out with a Pulfrich-photometer. Another experiment was carried out in the same way, but with alkali present in the inset to absorb the CO_2 produced so that the oxygen consumption could be measured. Controls without dye were run at the same time. Each dye was tested in different concentrations, and the concentration chosen for the final experiment, reported in the table, was the one in which the phosphate effect, i.e. synthesis of organic phosphate, if there was one, was best.

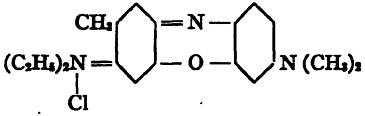
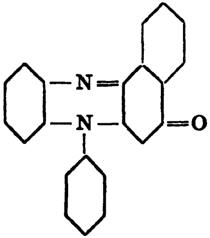
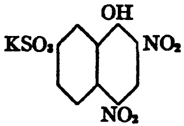
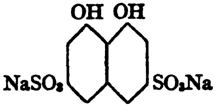
The type of curve obtained is shown, for a certain number of the dyes, in Figure 1.

Each vessel contained 0,5 cc Lebedev-saft + 0,5 cc aqueous dye solution of such concentration that the final concentration of dye was that shown below, + 0,1 cc of 40% glucose and 0,02 cc of 0,1 m potassium hexosediphosphate. The glucose and diphosphate were added from a side arm at time 0. At the time indicated by markers along the abscissa 0,1 cc of 0,1 M potassium hexosediphosphate was added from another side arm. In the oxygen uptake experiments the vessels also contained 0,2 cc NaOH in the inset. It should be noted that the O_2 scale is different from the CO_2 scale.

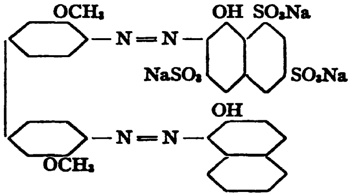
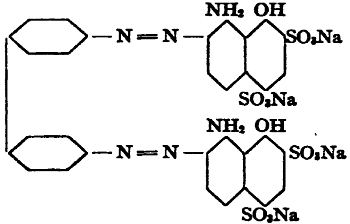
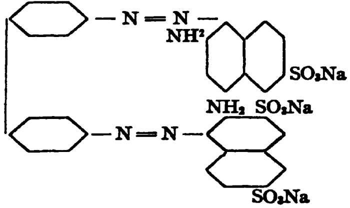
TABLE

Name of dye	Formula	Rowe's Color Index No.	Minimum concentration at which fermentation of glucose is entirely stopped	Ratio $\frac{CO_2 \text{ formed}}{P \text{ esterified}}$	Per cent of freshly esterified P which is hydrolyzed in 1 N H_2SO_4 in 1 hr.
1. 1-amino-2-naphthol-4-sulfonate		—	No inhibition up to 0,017 M. concentration		
2. Meldola's blue		909			
3. Indulin Scarlet B		827			
4. Nilblau		913			

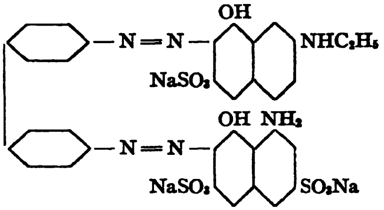
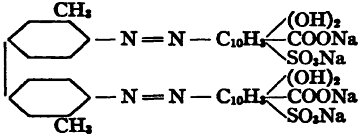
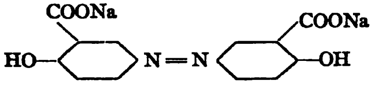
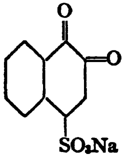
TABLE—*Continued*

Name of dye	Formula	Rowe's Color Index No.	Minimum concentration at which fermentation of glucose is entirely stopped	Ratio $\frac{CO_2 \text{ formed}}{P \text{ esterified}}$	Per cent of freshly esterified P which is hydrolyzed in 1 N H_2SO_4 in 1 hr.
5. Capriblau		876	No inhibition up to 0,017 M. concentration		
6. Rosindulin G	 An SO_3Na group is in the naphthalene nucleus	831			
7. 2—4 Dinitro-1 Naphthol-7-sulfonate		—	4,16 × 10^{-3} M		
8. 1—8 Dihydroxy-naphthalene 3—6-disulfonate		—	8,3 × 10^{-3} M		

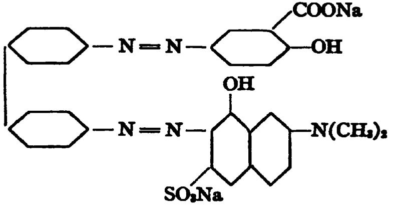
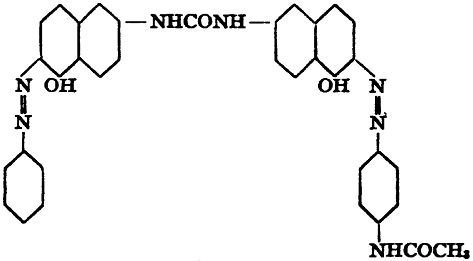
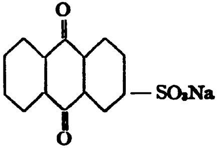
TABLE—*Continued*

Name of dye	Formula	Rowe's Color Index No.	Minimum concentration at which fermentation of glucose is entirely stopped	Ratio $\frac{CO_2 \text{ formed}}{P \text{ esterified}}$	Per cent of freshly esterified P which is hydrolyzed in 1 N H_2SO_4 in 1 hr.
9. Trisulfon Blue R......		464	8,3 × 10^{-4} M	1,28	12,0%
10. Evans Blue...........		Eastman Kodak M° 3873	8,2 × 10^{-4} M	1,18	9,7%
11. Brill. Congo Red R...		455	1,7 × 10^{-3} M	1,47	20,0%

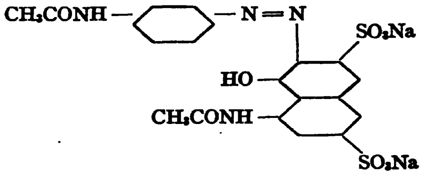
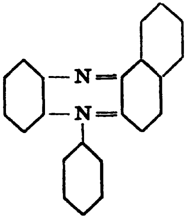
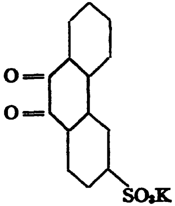
TABLE—*Continued*

Name of dye	Formula	Rowe's Color Index No.	Minimum concentration at which fermentation of glucose is entirely stopped	Ratio $\frac{CO_2 \text{ formed}}{P \text{ esterified}}$	Per cent of freshly esterified P which is hydrolyzed in 1 N H_2SO_4 in 1 hr.
12. Diphenyl Blue Black B D		402	2,1 × 10^{-3} M	3,23	8,4%
13. Direct Grey B		486	2,1 × 10^{-3} M	2,06	26,6%
14. Eriochrome Flavine A		219	4,2 × 10^{-3} M	2,03	5,1%
15. Sodium-beta-Naphthoquinone-4-Sulphonate		—	6,9 × 10^{-3} M	1,45	16,4%

TABLE—*Continued*

Name of dye	Formula	Rowe's Color Index No.	Minimum concentration at which fermentation of glucose is entirely stopped	Ratio $\frac{CO_2 \text{ formed}}{P \text{ esterified}}$	Per cent of freshly esterified P which is hydrolyzed in 1 N H_2SO_4 in 1 hr.
16. Diphenyl brown.......		422	8,3 × 10^{-3} M	2,41	5,7%
17. Benzo fast Scarlet 4 BS.		327	8,3 × 10^{-3} M	1,24	12,0%
18. Sodium anthraquinone-beta-sulphonate.......		—	8,3 × 10^{-3} M	1,18	10,7%

TABLE—*Concluded*

Name of dye	Formula	Rowe's Color Index No.	Minimum concentration at which fermentation of glucose is entirely stopped	Ratio $\frac{CO_2 \text{ formed}}{P \text{ esterified}}$	Per cent of freshly esterified P which is hydrolyzed in 1 N H_2SO_4 in 1 hr.
19. Amidonaphthol red....		57	1,7 × 10^{-2} M	1,26	18,0%
20. Rosindulin G G.......	 (an SO_3Na group is in one of the benzene nuclei)	830	4,9 × 10^{-3} M	1,00	20,2%
21. Phenanthraquinone-3 sulphonate...........		—	8,3 × 10^{-3} M	0,80	21,6%

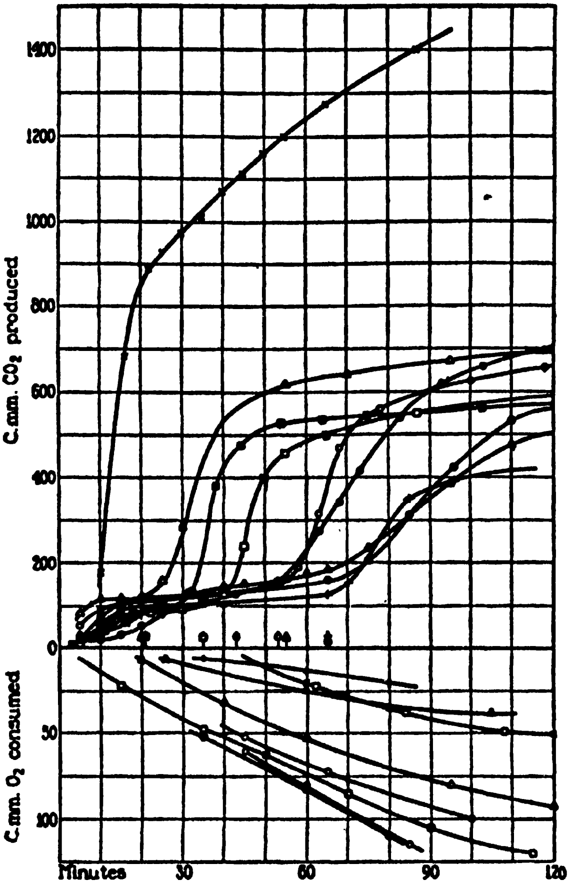


FIG. 1. The effect of various dyestuffs on the fermentation of glucose and of hexosediphosphate by yeast extract.

× represents the control
△ 8,3 × 10^{-3} M. Benzo-fast-scarlet
▬ 4,9 × 10^{-3} M. Rosindulin G G
☐ 8,3 × 10^{-4} M. Trisulfon blue
○ 2,1 × 10^{-3} M. Direct grey B.
● 8,2 × 10^{-4} M. Evans Blue
× 8,3 × 10^{-3} M. Sodium anthraquinone B-sulfonate
▲ 1,7 × 10^{-3} M. Brilliant Congo red R.
+ 4,2 × 10^{-3} M Eriochrome flavin A.

DISCUSSION

As in the previous paper one can easily distinguish three groups of dyes. The first group consists of those dyes which do not inhibit the fermentation of glucose to any appreciable extent, even in high concentration. Examples of these are the first six dyes listed in the table. The second group inhibits or stops fermentation as a whole. Examples are Nos. 7 and 8 in the table. The third group consists of dyes which inhibit fermentation of glucose but not of diphosphate. Examples are Nos. 9–21 inclusive. Within this group there are dyes which exhibit a complete phosphate effect, i.e., which cause the synthesis of one molecule of difficultly hydrolyzable phosphate ester from inorganic phosphorus for each molecule of CO_2 formed from the hexosediphosphate added. There are other dyes with an incomplete phosphate effect, i.e. although they inhibit fermentation of glucose, and not that of hexosediphosphate, they cause a synthesis of a smaller amount of difficultly hydrolyzable phosphate than corresponds to the CO_2 developed.

Another division of the dyes exhibiting the phosphate effect that can be made is that one group causes an increase in oxygen uptake, such as is exhibited also by many other dyes not showing either inhibition or phosphate effect, and another group does not cause an increased oxygen uptake. The effect on oxygen uptake is clearly not at all parallel to the specific inhibiting effect. The dyes like phenanthrene quinone 3-sulfonate and beta-naphtoquinone-4-sulfonate which resembles rosindulin G G (also quoted as rosindulin 2G) with respect to inhibition and phosphate effect, increase the oxygen consumption to as great an extent as does methylene blue. The latter, however, shows no similarity in its inhibiting effect to rosindulin. It is surprising that rosindulin G, which, according to the literature as quoted in Rowe's Color Index (830 and 831), differs from G G only by the position of the SO_3H group, and which, with respect to the oxidation-reduction potential, the color and the lack of any influence upon oxygen consumption can scarcely be distinguished from rosindulin G G, shows practically no inhibition of the fermentation of glucose in any way. These two very similar dyes show with respect to the degree and manner of the inhibiting effect the greatest possible contrast.

In the table the column indicating the ratio of CO_2 formed to P esterified is of especial interest. If this ratio is 1, the effect is highest; for each molecule of CO_2 appearing one molecule of inorganic phosphate is esterified. When this ratio is greater, the specific effect is incomplete. Of the thirteen dyes listed which show the effect at all, only one has a ratio as high as 3,0. Three others are 2,0 or above and the rest are under 1,5.

The last column indicates the extent to which the organic phosphate formed is readily hydrolyzed The highest value found for one hour hydrolysis in 1.0 N H_2SO_4 at 100°C is 26.6%. Such figures indicate that most of the ester belongs to the difficultly hydrolyzable fraction (the monophosphate fraction (2)).

SUMMARY

The effect of twenty-one dyes on fermentation by yeast extract has been tested. Of this number six dyes showed no inhibiting effect, two showed a general inhibiting effect and thirteen inhibited the fermentation of glucose completely, but did not inhibit the fermentation of hexosediphosphate. In the latter group the fermentation of the diphosphate is accompanied by a synthesis of organic phosphate from the inorganic phosphate present. The amount of this synthesis varies from 1 atom of phosphorus per molecule of CO_2 formed to about 1 phosphorus per three CO_2. The oxygen consumption caused by the dye does not parallel the effect of the dye on fermentation. No definite correlation between chemical structure and effect of the dyes can be observed.

1) L. Michaelis, C. V. Smythe, Jl. of Biol. Chem. **113**, 717 (1936).—2) C. V. Smythe, ibid. **118**, 619 (1937).—3) O. Meyerhof, W. Kiessling, Bioch. Zs. **281**, 249 (1935).—4) K. Kottmann, Schweiz. med. Ws. **16**, 533 (1935).

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TRANSFERENCE NUMBERS AND ION MOBILITIES OF SOME ELECTROLYTES IN DEUTERIUM OXIDE AND ITS MIXTURES WITH WATER

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INTRODUCTION

In previous publications from this Laboratory, transference data obtained by the method of moving boundaries,[1] of many of the strong electrolytes have been reported. These data are of value, in the first place, in the accurate determination of the activities of electrolytes from potential measurements on concentration cells with transference[2,3] and, in addition, they may be combined with conductance measurements to give ionic mobilities as functions of the concentration.[4] It is our program to extend such measurements to other solvents. Heavy water is of particular interest since, of the variables entering into modern solution theory, its viscosity is the only property in which it differs appreciably from normal water.[5] Moreover, we believe that the data to be presented in this paper will eventually be of service in explaining the abnormal mobilities of the hydrogen and deuterium ions.

EXPERIMENTAL PROCEDURE

Since the moving boundary method for the determination of transference numbers normally requires only small volumes of solution it is quite suitable for work with limited amounts of material. With the apparatus previously described[1] and using autogenic boundaries only 15 ml. of solution was required for a determination. This quantity was further reduced to 10 ml. by redesigning the elec-

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(1) MacInnes and Longsworth, *Chem. Rev.*, **11**, 171 (1932).

(2) Brown and MacInnes, THIS JOURNAL, **57**, 1356 (1935).

(3) MacInnes and Brown, *Chem. Rev.*, **18**, 335 (1936).

(4) MacInnes, Shedlovsky and Longsworth, THIS JOURNAL, **54**, 2758 (1932).

(5) Jones and Fornwalt, *J. Chem. Phys.*, **4**, 30 (1936).

trode chamber as shown in cross section in Fig. 1. The function of the inner cup A is to keep the products of the electrode reaction, $AgCl + \epsilon^- = Ag + Cl^-$, from altering the composition of the solution at the boundary in the graduated tube T. The silver-silver chloride cathode was in the form of a flat spiral resting on the bottom of the inner cup so that the concentrated chloride solution which forms at this electrode during an experiment tends to remain in place. Moreover, by constricting the lower end of the glass tube B the solution in the bottom of the cup tends to remain cooler than in the upper part and thus further restricts convection.

The conductivity cell used was of the pipet type. The electrodes were truncated hollow cones of platinum, the outer surfaces of which are completely sealed to the glass, as described by Shedlovsky.[6] It had a capacity of 4 ml. and a constant of 16.482 on the basis of a specific conductance of 0.012856[7] for 0.1 demal potassium chloride at 25°. With the solutions whose conductances were measured in this cell the error due to the Parker effect[8] was never greater than 0.03%, which was about the accuracy with which the concentrations of the solutions were known.

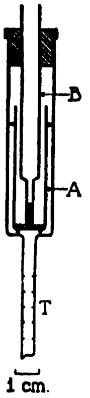

FIG. 1.—Modified electrode chamber

Densities were determined with the aid of a pycnometer similar to that described by Smith and Wojciechowski.[9] The volume of the pycnometer was 8.5 ml. and the capillary neck, marked with a single graduation, had an internal diameter of 1.4 mm. The pycnometer was filled with the aid of a hollow stainless steel needle. It was supported in a thermostat at 25° on a framework which also carried, outside the glass wall of the thermostat, a microscope whose position

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(6) Shedlovsky, THIS JOURNAL, **54**, 1411 (1932).

(7) Jones and Bradshaw, *ibid.*, **55**, 1780 (1933).

(8) Parker, *ibid.*, **45**, 1366, 2017 (1923).

(9) Smith and Wojciechowski, *Roczniki Chem.*, **16**, 104 (1936).

could be shifted and read with a micrometer screw. When observation with the microscope indicated that the meniscus had stopped moving, its position relative to the graduation was read to 0.001 cm. Weighings were then made with a duplicate pycnometer as counterpoise. An air density of 0.0012 was used in the reduction of all weights to vacuum. It may be noted that the densities of light and heavy water result in appreciably different vacuum corrections.

The samples of sodium and potassium chlorides were purified by recrystallization and fused prior to weighing. In the preparation of the hydrogen-deuterium chloride solutions constant boiling hydrochloric acid, whose concentration was checked at intervals by conductance measurements, was added to the solvent from a small weight buret. The solvent was distilled under its own vapor pressure from an alkaline permanganate solution at 40° into a receiver at 0°. If precautions were taken to prevent ebullition, a single distillation was sufficient to give water having a specific conductance less than 10^{-6} mhos and no water was used as solvent having a conductance greater than that value. About 20 ml. of each solution was prepared. The moving boundary cell, conductivity cell and pycnometer, previously cleaned and dried, were filled without rinsing.

EXPERIMENTAL RESULTS

The experimental results consist (a) of a study of the transference numbers and conductances of 0.05 normal solutions of potassium chloride in a wide range of mixtures of hydrogen and deuterium oxides, (b) similar measurements on 0.05 normal solutions of hydrogen–deuterium chloride and (c) measurements of three strong electrolytes over a range of salt concentrations in nearly pure deuterium oxide.

(*a*) *Solutions of Potassium Chloride in H_2O–D_2O Mixtures.*—The results of measurements of solutions of potassium chloride in H_2O–D_2O mixtures are recorded in Table I. The concentrations, in equivalents per liter of solution at 25°, are given in the first column. The mole fraction of deuterium oxide in the solvent, N_{D_2O}, is recorded in the second column. This was computed directly from the weights of hydrogen and deuterium oxides or from the measured density of the mixture, using the relation[10]

$$N_{D_2O} = \frac{9.2351\ \Delta S}{1 - 0.0327\ \Delta S}$$

in which $\Delta S = (d^{25}{}_4/0.99705_6) - 1$, obtained from recent measurements made in this Laboratory. Columns 3, 4 and 5 of Table I contain,

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(10) Longsworth, THIS JOURNAL, 59, 1483 (1937).

respectively, the observed density of the solution, the equivalent conductance and the cation transference number. The data for normal water in this and later tables are from earlier work from this Laboratory.[6,11] The necessary small solvent and volume corrections[1] have been applied to the transference data. In making the volume corrections the molal volumes of the electrolytes were assumed to have the same values in the mixtures as in water, an assumption that is justified by existing density data of solutions in deuterium oxide.

TABLE I

Transference Numbers and Conductances of 0.05 Normal Solutions of KCl in Mixtures of D_2O and H_2O at 25°

1	2	3	4	5	6	7	8
Concn.	N_{D_2O}	d^{25}_{4}	Λ	T_+	T_+ computed equation (1)	λ_{Cl}	λ_{Cl} computed equation (2)
0.04997	0.9935	1.10614	110.51	0.4939	0.4939	55.93	55.93
.05007	.7521	1.08027	115.33	.4932	.4929	58.45	58.46
.05002	.4959	1.05277	120.89	.4916	.4919	61.46	61.43
.04999	.2471	1.02610	126.86	.4909	.4909	64.58	64.60
.05000	.0002	0.99942	133.37	.4899	.4899	68.03	68.03

The transference numbers given in Table I for 0.05 normal solutions of potassium chloride in H_2O–D_2O mixtures vary linearly with the mole fraction, N_{D_2O}. Values of T_+ computed from the relation

$$T_+ = 0.4899 + 0.0040\, N_{D_2O} \qquad (1)$$

are recorded in column 6 of Table I and agree with the observed values within the experimental error. Theory,[11] and the experimental results given below in Table III, both indicate that transference numbers of potassium chloride change but little with salt concentration so that the variation with N_{D_2O} given by equation (1) is valid for solutions at infinite dilution. It may be shown readily that this variation of the transference number with change of solvent is contrary to the assumptions underlying the Walden rule,[12] *i. e.*, $\Lambda_0\eta$ = constant, in which η is the viscosity of the solvent. Since the ions are assumed to move

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(11) Longsworth, *ibid.*, **54**, 2741 (1932).

(12) Walden, *Z. physik. Chem.*, **55**, 207 (1906). See also Ulich, *Fortschr. Chem., Physik physik. Chem.*, **18**, 567 (1926).

independently and Stokes' law is assumed to apply to each, then for a binary electrolyte $\lambda_0^+\eta = k^+$ and $\lambda_0^-\eta = k^-$, in which λ_0^+ and λ_0^- are the limiting ion conductances and k^+ and k^- are constants. From these relations $T_0^+ = \lambda_0^+/(\lambda_0^+ + \lambda_0^-) = k^+/(k^+ + k^-)$. Thus it is a corollary of the Walden rule that the limiting transference number should be independent of the solvent, which, as has been shown from the data in Table I and equation (1), is not true for solvents as similar as hydrogen and deuterium oxides.

The conductances of the chloride ion constituent, which is common to all of the electrolytes considered in this paper, are recorded in the seventh column of Table I. These data may be represented adequately by the relation

$$\lambda_{\text{Cl}}(0.05\ N\ \text{KCl}) = 68.03 - 14.46\ N_{\text{D}_2\text{O}} + 2.30\ N^2_{\text{D}_2\text{O}} \quad (2)$$

as is indicated by the agreement between the values of columns 7 and 8.

TABLE II

Transference Numbers and Conductances of 0.05 Normal Solutions of HCl–DCl in Mixtures of D_2O and H_2O at 25°

1	2	3	4	5	6	7	8
Concn.	$N_{\text{D}_2\text{O}}$	d^{25}_4	Λ	$\Lambda(0.05\ N)$	T_+	T_+ compd.	ΔT_+
0.05464	0.9853	1.10400	290.9_8	291.6_8	0.8081	0.8077	−0.0002[a]
.05086	.9007	1.09481	295.5_0	295.6_4	.8065	.8073	+ .0008
.04966	.8082	1.08499	301.1_9	301.1_1	.8072	.8076	+ .0004
.05049	.5990	1.06243	317.3_7	317.4_6	.8094	.8101	+ .0007
.05013	.4052	1.04167	338.1_2	338.1_4	.8146	.8147	+ .0001
.05073	.1972	1.01931	366.3_3	366.4_7	.8217	.8216	− .0001
.05000	.0002	0.99798	399.0_8	399.0_8	.8292	.8292	.0000

[a] T_+ obsd. = 0.8079 at C = 0.050.

(*b*) *Solutions of Hydrogen–Deuterium Chloride in H_2O–D_2O Mixtures.*—The results of measurements on 0.05 normal solutions of hydrogen–deuterium chloride in several H_2O–D_2O mixtures are recorded in Table II. In the computation of $N_{\text{D}_2\text{O}}$ for these solutions account was taken of the water added in the form of constant boiling hydrochloric acid but the very slight change in $N_{\text{D}_2\text{O}}$ due to the reaction[13]

$$2\text{H}^+ + \text{D}_2\text{O} = 2\text{D}^+ + \text{H}_2\text{O} \quad (3)$$

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(13) Korman and La Mer, THIS JOURNAL, 58, 1396 (1936).

was ignored. The observed conductances and transference numbers are recorded in columns 4 and 6, respectively. Since the concentrations of some of the solutions differed from 0.05 it was desirable to adjust the conductances to this concentration. This was done with more than sufficient accuracy with the aid of the Shedlovsky equation,[14] the coefficient B of that equation being set equal to $(\alpha\Lambda_0 + \beta)$. The adjusted values are recorded in column 5 of Table II. If these equivalent conductances are plotted against the mole fraction of the solvent, N_{D_2O}, the resulting curve sags below the straight line connecting the limiting values in the pure solvents, an observation also made by Baker and La Mer[15] on 0.02 normal solutions of hydrogen and deuterium chlorides. Assuming that the chloride ion conductances of these solutions are normal, values of T_+, column 7, were computed by means of the relation

$$1 - T_+ = \frac{\lambda_{Cl}(0.05\ N\ KCl)}{\Lambda(0.05\ N\ HCl\text{-}DCl)} + 0.0003$$

in which λ_{Cl} is given by equation (2), Λ is taken from column 5 of Table II and the small constant term corrects for the slight difference in the chloride ion conductance in potassium and hydrogen chloride solutions. The agreement between the observed and computed values of T_+ is sufficiently close to justify the assumption on which the equation just given is based.[16] The cation constituents of the hydrogen–deuterium chloride solutions must therefore be responsible for the large deviations from linearity mentioned above.

(14) Shedlovsky, THIS JOURNAL, **54**, 1405 (1932).

(15) Baker and La Mer, *J. Chem. Phys.*, **3**, 406 (1935).

(16) The differences at $N_{D_2O} = 0.9$, 0.8 and 0.6 are probably outside of the experimental error and may be due to the complex nature of the cation constituents in these mixtures. It was observed that the boundaries became less sharp as they moved and in some cases a progressive separation into two boundaries was observed. A possible explanation is as follows. More hydrogen than deuterium ions leave the region immediately ahead of the boundary. Thus the equilibrium indicated by equation (3) must be displaced to the left to compensate for the loss of hydrogen ion. The resulting shift in the composition of the solvent in the neighborhood of the boundary may therefore account for the complications observed at these boundaries. A computation indicates that although a measurable accumulation of deuterium oxide occurs behind the boundary it is of no practical importance.

Baker and La Mer[16] explain, qualitatively, the irregular behavior of the cation constituents of hydrogen–deuterium chloride solutions as follows. They accept the hypothesis, treated theoretically in several recent papers,[17] that the high mobilities of the hydrogen and deuterium ions in hydrogen and deuterium oxides, respectively, are due in a large measure to symmetrical transitions of the type

$$H_3O^+ + H_2O = H_2O + H_3O^+$$
$$D_3O^+ + D_2O = D_2O + D_3O^+$$

In H_2O–D_2O mixtures, however, the foregoing transitions are partially replaced by unsymmetrical, and hence less probable, transitions of which the following is an example

$$D_3O^+ + H_2O = D_2O + DH_2O^+$$

Since the total number of possible transitions in a mixture is essentially the same as in the pure solvent, any decrease in the probability of certain types of transitions will be accompanied by a corresponding decrease in the apparent mobility of the hydrogen and deuterium ions. Our transference number measurements in these mixtures are compatible with this hypothesis since it attributes all abnormalities to the cation constituents and permits the chloride ion to be normal. Of the various hypotheses that may be advanced in explanation of the observed cation conductances of hydrogen–deuterium chloride in H_2O–D_2O mixtures, one which our data definitely excludes is that the negative deviations are due to ion association. With this hypothesis the chloride ion conductance in these mixtures would be less than in other chloride solutions at the same concentration of electrolyte and mole fraction of deuterium oxide and this is not observed.

(*c*) *Solutions of Sodium, Potassium and Deuterium Chloride in Heavy Water.*—Transference number and conductance measurements on solutions of three chlorides in nearly pure deuterium oxide are reported in Table III. These data may be interpreted with the aid of a quantity, λ_0', defined by the relation

$$\lambda_0' = (\lambda + \beta\sqrt{C})/(1 - \alpha\sqrt{C})$$

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(17) Hückel, *Z. Elektrochem.*, **34**, 546 (1928); Bernal and Fowler, *J. Chem. Phys.*, **1**, 515 (1933); Wannier, *Ann. Physik.* **24**, 545, 569 (1935).

in which α and β are constants of the Onsager theory and have the values 0.2323 and 24.53, respectively, for D_2O at 25°.[5,18] Values of λ_0 for the four ion constituents of the electrolytes of Table III are plotted as ordinates against the concentration as abscissa in Fig. 2. As in water[4] λ_0' is found to be a linear function of the concentration for each ion species, *i. e.*

$$\lambda_0' = \lambda_0 + bc$$

and this relation may be used to obtain values of the ion conductance at zero concentration, λ_0. Values of λ_0 and b, as computed by the method of least squares, are recorded in Table IV. The average value of λ_0 for the chloride ion from the three chlorides of Table III is 62.7_9 with an average deviation from the mean of 0.07%. It is of interest that the values of b for this constituent show a parallel but somewhat smaller variation than is found with the data for water solutions.

TABLE III

Transference Numbers and Conductances of Some Electrolytes in Heavy Water at 25°

1	2	3	4	5	6
Salt	Concn.	N_{D_2O}	d^{25}_4	Λ	T_+
KCl	0.02013	0.9935	1.10485	114.53	0.4940
	.04997	.9935	1.10614	110.51	.4939
	.09985	.9935	1.10843	106.87	.4939
NaCl	.02013	.9935	1.10473	95.50	.3922
	.04996	.9938	1.10596	91.74	.3901
	.09735	.9938	1.10791	88.36	.3877
HCl–DCl	.02129	.9889	1.10382	297.8_0	.8055
	.05464	.9853	1.10400	290.9_6	.8081
	.10182	.9773	1.10391	286.2_4	.8105

The values of λ_0 recorded in Table IV are limiting ion conductances in an H_2O–D_2O mixture for which $N_{D_2O} = 0.9935$. This value for the mole fraction of deuterium oxide is based upon the density data for the pure material given by Selwood, Taylor, Hipple and Bleakney.[19]

(18) Müller, *Physik. Z.*, **35**, 1009 (1935).

(19) Selwood, Taylor, Hipple and Bleakney, This Journal, **57**, 642 (1935).

In view of the existing uncertainty[20] as to the density of pure deuterium oxide, it is not desirable at present to extrapolate our ion conductances to their values for the pure solvent. The extrapolation will, in any event, be a very short one and can be made readily from the data of this paper when the density of pure deuterium oxide has been determined.

It is of interest that our data for the chloride ion constituent of potassium chloride give the nearly identical values of 1.1221 and 1.1218 for the ratio $\lambda_{Cl}(C = 0)/\lambda_{Cl}(0.05\ N\ \text{KCl})$ in light and heavy water, respectively. La Mer and Chittum[21] have shown recently from theory and experiment that the salt conductance ratio Λ_0/Λ_c is very nearly constant for these two solvents and their mixtures. We thus have

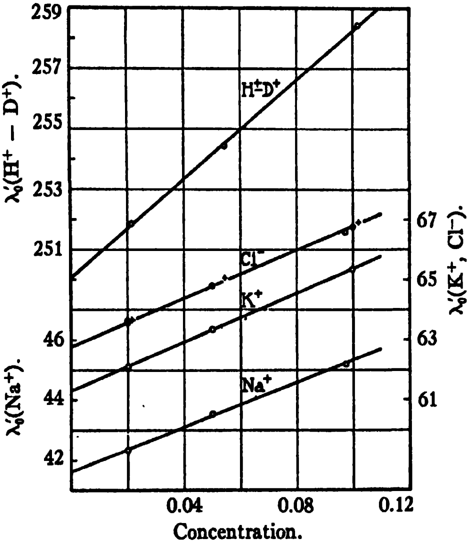

FIG. 2

$$\lambda_0(N_{D_2O}) = (\lambda_0/\lambda_c)_{H_2O}\,\lambda_c(N_{D_2O}) \qquad (4)$$

in which λ_c and λ_0 are the conductances of a given ion constituent at concentration C and at infinite dilution, respectively. Substitution in

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(20) Tronstad, Nordhagen and Brun, *Nature*, **136**, 515 (1935).

(21) La Mer and Chittum, THIS JOURNAL, **58**, 1642 (1936).

equation (4) of the value for $\lambda_0(N_{D_2O})$ given for the chloride ion by equation (2) and 1.1221 for the ratio $(\lambda_0/\lambda_0)_{H_2O}$ gives

$$\lambda_0(Cl^-) = 76.34 - 16.23\ N_{D_2O} + 2.58\ N^2_{D_2O}$$

a relation that may be used in determining the limiting transference number of any chloride in any H_2O–D_2O mixture for which the limiting salt conductance is known.

TABLE IV

Limiting Ion Conductances in Heavy Water at 25°

Salt	Ion	*b*	λ_0
KCl	K^+	40.33	61.31
NaCl	Na^+	36.73	41.64
HCl-DCl[a]	H^+–D^+	82.18	250.0_5
KCl	Cl^-	40.19	62.74
NaCl	Cl^-	38.27	62.86
HCl-DCl[a]	Cl^-	40.64	62.79
		Average	62.7_9

[a] In the computation of λ_0' for the ion constituents of the hydrogen–deuterium chloride solutions it was necessary to correct the observed conductances to their values for $N_{D_2O} = 0.9935$. These small corrections were made as follows. The conductance data of Table II may be represented by the empirical equation $\Lambda(0.05\ N\ \text{HCl-DCl}) = 399.11 - 179.5\ N_{D_2O} + 71.8\ N^2_{D_2O}$ with an average deviation of 0.05%. Assuming that the value of $d\Lambda/dN_{D_2O}$ obtained by differentiating this expression is also valid at concentrations in the neighborhood of 0.05 N, the value of the necessary correction, $\Delta\Lambda$ was computed from the relation $\Delta\Lambda = (-179.5 + 143.6\ N_{D_2O})(0.9935 - N_{D_2O})$.

The authors wish to thank Professor La Mer and Dr. Chittum of Columbia University for many helpful suggestions concerning experimental technique.

SUMMARY

Transference and conductance measurements on solutions of several electrolytes in deuterium oxide and its mixtures with water have been made. The investigation included 0.05 N solutions of potassium and hydrogen–deuterium chlorides in a wide range of H_2O–D_2O mixtures. The conductance of the chloride ion constituent from these two

chlorides varies nearly linearly with the mole fraction of deuterium oxide in the mixed solvent. The hydrogen–deuterium component, on the other hand, shows wide variations from linearity, thus indicating a complex conductance mechanism.

Measurements have also been made on solutions of sodium, potassium and hydrogen–deuterium chlorides at several concentrations in nearly pure ($N_{D_2O} = 0.9935$) deuterium oxide. From these data limiting values of the ion conductances have been computed for heavy water.

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THE CULTURE OF WHOLE ORGANS

II. The Effects of Perfusion on the Thyroid Epithelium

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Plates 4 and 5

(Received for publication, June 1, 1937)

With the possibility of cultivating whole organs in the Lindbergh apparatus (1) a new field in experimental biology has been opened up. During the last year numerous experiments with culture of the isolated thyroid gland have been carried out in this laboratory. The technique of operation and cultivation has been described in a previous paper (2). The present report deals with the morphological findings in more than a hundred cat and rabbit thyroids which have been cultivated for various lengths of time.

Technical

The animals were etherized and the thyroid gland dissected and inserted into the organ chamber of the Lindbergh apparatus as described by Carrel (2). Either the right lobe was isolated for cultivation, while the left lobe was saved for immediate histological cultivation; or both lobes were dissected and cultivated separately. Generally, the culture medium contained 20 per cent homologous serum diluted with glucose-Tyrode solution; 5 mg. per cent phenol red was added as indicator. In many cases 40 pet cent serum was used. As soon as the perfusion was stopped, the glands were removed and fixed for histological examination. Maximov's Zenker-formol solution was used as the standard method for fixation. The material was embedded in paraffin, cut in short series at 5 microns and stained with hematoxylin-eosin, Heidenhain's iron-hematoxylin and sometimes Bensley's and Masson's stains. In a number of cases small pieces were fixed and saturated according to the Ludford modification of the Mann-Kopsch osmic acid technique. The Da Fano silver impregnation has been utilized but did not prove satisfactory.

Effects of Perfusion on the Thyroid Epithelium

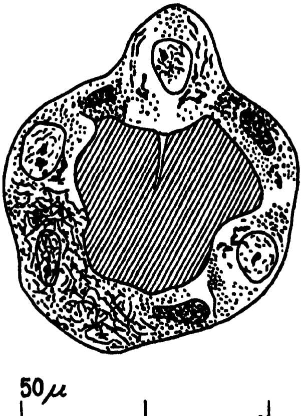
During cultivation the thyroid gland does not show appreciable macroscopical changes, provided the circulation is kept at a normal rate and no accidents occur. In the areolar connective tissue which envelops the gland, a considerable edema may develop due to oozing collaterals; but neither does this seem to affect the gland, nor is it accompanied by any edematous state of the thyroid tissue itself.

Microscopically, the gland may remain normal or show areas in which the cells are shrunken, the nuclei pycnotic and the general aspect that of a necrobiotic state of the tissue. Such alterations are not uncommon. They may appear only as small spots, or they may take up a considerable part of the total amount of parenchyma. In the more extensive form, this condition is, however, of exceptional occurrence. We have not been able to trace the cause of the damaged state of small areas of the tissue. It is possible that minute particles or small air bubbles which have been able to pass the filters, are responsible for the appearance of such degenerated spots by causing small emboli. Anyway, the presence of such partial alterations does not seem to influence the morphology of the remaining tissue.

In our attempt to describe the histological and cytological aspect of thyroid glands after cultivation, we have found it convenient to arrange the material in groups: glands cultivated for less than 24 hours; glands cultivated for 24 to 48 hours; glands cultivated for about 1 week; and glands cultivated for more than 1 week.

Cultivation for only a few hours will not bring about variations in the form and size of the thyroid vesicles. The colloid is as a rule not affected, its stainability and density remain unaltered and the number and size of vacuoles are practically the same as in the control glands. The cells in some vesicles may show a very slight swelling, but this is by no means a constant finding. As the structural aspect of the cytoplasm is essentially the same whether the duration of the experiment is 4 or 24 hours, we shall reserve the descriptions of these findings for the next group.

In glands which have been cultivated over periods of between 24 and 48 hours, the form and size of the vesicles is the same as in the control glands. The colloid is of the same density and stainability, and the amount of vacuolization is only slightly increased. This change is, however, not at all constant and it is not present in all vesicles. The vacuolization seems to be less liable to occur in the larger vesicles with dense colloid and flattened cells than in the smaller units. The epithelium in a cultivated gland is generally slightly higher than in the control. But again, this change is not taking place uniformly throughout the parenchyma. And again, the smaller vesicles, and especially those in the central part of the gland, are more liable to show the reaction than are the larger ones. This cellular swelling is an increase in volume which is different from the edematous state which may precede cellular degeneration. It is much more like the initial swelling of the thyroid cells which is observed during the first stages of experimentally increased secretory activity. Not all the thyroid cells along the periphery of any given vesicle show the same degree of swelling. Most commonly, only a few cells wide apart will react; in vesicles where most of the cells are cuboidal or flat, the contrast between the swollen clear cells and their more deeply stained neighbors may be very striking (Text-fig. 1).



TEXT-FIG. 1. Vesicle from rabbit thyroid gland, perfused for 24 hours with 40 per cent rabbit serum in glucose-Tyrode solution, showing considerable swelling of a single epithelial cell. Osmic impregnation; Heidenhain's iron-hematoxylin stain.

The mitochondria are either of the filamentous or of the short rod-like type (Fig. 6). They are mostly well conserved and normally stainable in the preparations from glands cultivated for 48 hours. The distribution and size correspond well with what is found in the uncultivated gland from the same animal. The Golgi apparatus of the thyroid cells in glands perfused from 24 to 48 hours is reticular in shape and its localization and general structure is the same as in the control glands (Fig. 3). It is interesting to note that precisely in the type of swollen cells mentioned above, the Golgi apparatus is expanded and partially disintegrated in a manner which closely resembles that which is observed during the first phase of stimulated activity of the thyroid cells. The reticular material is much looser than usual and small fragments of the filaments—not granules—are floating in the clear swollen cytoplasm of the supranuclear region of the cell.

The blood vessels and the mesenchyme do not show any sure signs of alterations due to perfusion during 1 or 2 days.

Cultivation of the thyroid gland for about 1 week need not affect the general histology of the gland. The size of the vesicles may show a tendency to increase. The colloid may show a somewhat higher degree of vacuolization and, in some vesicles, a different stainability compared with the lobe fixed immediately after the death of the animal. Generally, the gland is in a morphologically good condition. Cytological examination reveals, however, that structural changes have actually taken place during the experiment. The mitochondria are mostly granular or indistinct, and the Golgi apparatus does not show a filamentous or reticular shape any longer; instead, an accumulation of osmiophile droplets is seen to coincide with the Golgi zone of the cytoplasm. It seems as if the finer cytoplasmatic structures gradually undergo degeneration during the last part of the week's perfusion. It should be noted, however, that these changes do not interfere with the life of the cells; positive epithelial cultures can be obtained from thyroids perfused for 18 or 21 days. Possibly the violent osmotic changes and the coagulation due to fixation are liable to cause more considerable disturbances in the cytoplasm exposed for several days to artificial and not entirely adequate nutritional conditions than is the case during the usual process of fixation in tissues taken immediately from the animal. The blood vessels do not show sure signs of alterations. But the mesenchyme may show signs of proliferation, the degree and type of which is, however, highly variable.

Cultivation of the thyroid gland for several weeks is possible when the gland is transferred every week to another pump with a fresh supply of perfusion fluid. After 1 month, the thyroid tissue may retain the histological features of a normal gland. The epithelium seems to assume an aspect of inactivity, the cells are flattened, and the colloid may be very dense and abundant (Fig. 4). The number of such experiments of long duration is, however, too small to enable us to put forward any complete statement of the morphology of this particular group.

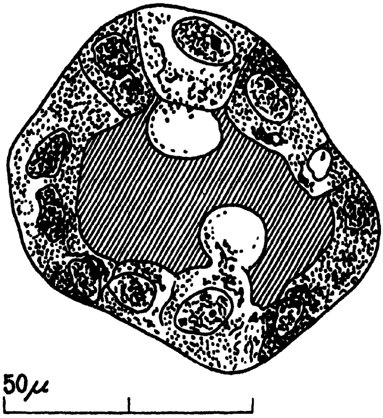
Because of the considerable variability of the structure of cat and rabbit thyroid glands, both lobes should always be matched in experiments with culture of the isolated gland; one side to be used for the experiment, the other as a control. The fact that the process of perfusion is liable to bring about certain structural changes in the thyroid epithelium makes such an arrangement even more important.

Effects of Stimulation on Isolated Thyroid Glands

The process of cultivation, and especially changes in the composition of the perfusion fluid (notably the admixture of certain substances to the medium, as for example Witte's peptone), has the effect of inducing to a more or less marked degree an increase and probably a stimulation of the secretory elements. Biochemically, certain changes in the secretory activity can be detected. The results of the iodine analysis of different glands and the corresponding perfusion fluids will be published later.

As a method of stimulating the thyroid cells under well known experimental conditions, we have utilized the thyro-stimulating factor from the anterior pituitary. We have used the preparation of Collip, commercially prepared by Ayerst, McKenna and Harrison. The preparation was first tested on guinea pigs and we satisfied ourselves that a dose of 1 cc., injected subcutaneously, was able to release a very marked histological and cytological reaction in the thyroid gland of these animals. A great number of cat and rabbit thyroid glands were then cultivated under various experimental conditions in order to observe the effect of the pituitary stimulation upon isolated thyroid glands. Either the thyro-stimulating factor was added to the medium at the beginning of the experiment, or the gland was cultivated for 24 hours and then the dose of thyro-stimulating factor was added. Or else pituitary extract was added at once and then, after 24 hours, another dose was added to the medium. The amounts utilized have been 1 to 2 cc. The duration of the experiments has been from 3 hours to 6 days. While one lobe has been cultivated for the experiment, the other lobe has always been perfused in another pump as a control.

Generally, the response of the thyroid epithelium to the stimulation has been similar, if not identical, to that observed in the living animal. In the preparations from glands cultivated for 24 hours, in which the thyro-stimulating factor has been added at the beginning of the experiment, there is a considerable vacuolization of the colloid, its stainability is often diminished and the size of the vesicles seems to be slightly decreased. There is, as a rule, a considerable swelling of the supranuclear regions of most of the thyroid cells (Fig. 5), and mitotic activity may be observed (Figs. 1 and 2). The mitochondria are increased in size and definitely filamentous. The Golgi apparatus is increased in size and is often of a distinctly reticular shape. Vacuoles appear both in the Golgi zone and in the basal regions of the epithelial cells (Text-fig. 2).



TEXT-FIG. 2. Vesicle from rabbit thyroid gland, perfused for 24 hours with 40 per cent serum in glucose-Tyrode solution plus 0.8 per cent anterior pituitary extract, showing vacuolization of colloid, swelling of epithelial cells and increase of the Golgi apparatus. Osmic impregnation; Heidenhain's iron-hematoxylin stain.

There are, however, differences between the morphological aspect of pituitary thyro-stimulation during perfusion and the corresponding reaction in the living animal. First, the reaction in the isolated gland is never so pronounced as in the gland *in situ*; the degree of the cellular response and the uniformity of the tissue affected is inferior in the case of the cultivated glands. Next, there is a difference in time. In the living animal the thyro-stimulation with the pituitary factor takes place very quickly, so that the tremendous cytoplasmatic swelling which is characteristic of the initial stage of the reabsorptive phase of thyroid activity, occurs inside the 1st hour after stimulation; and during the following hour the intense vacuolization and release of the colloid material is evident. In the culture experiments, the cellular reactions may be comparatively slight, even 3 or 4 hours after the introduction of the thyro-stimulating factor. 6 to 8 or 10 hours later, the cellular stimulation is fairly well established. The state of morphologically increased secretory activity is, as a rule, plainly evident during the next 1 or 2 days. Even in experiments in which the thyroid gland has been cultivated for 6 days, there may be a considerable difference between the stimulated and the control gland. But the reaction does at that time show a tendency to diminish. Whether this is due to disappearance of the thyro-stimulating factor; whether it is a sign of exhaustion on the part of the thyroid cells; or whether the slowing down of the reaction is caused by an accumulation, in the medium, of active iodinized compounds, which in turn may react upon the thyroid epithelium and thus cause a differential mechanism, antagonizing the action of the pituitary factor, cannot be determined at present. Specially designed experiments are needed to elucidate this particular problem. But there is a definite response to the thyro-stimulating pituitary factor on the part of the thyroid epithelium, and the reaction seems to be fundamentally the same as that observed in the living animal.

CONCLUSIONS

1. During the 1st and 2nd day of cultivation of the whole thyroid gland in the Lindbergh apparatus, perfused by a medium containing 20 or 40 per cent homologous serum diluted with glucose-Tyrode, the state of the thyroid epithelium remains histologically normal and even the most delicate cytological details retain their normal aspect.

2. Cultivation over a period up to 6 days need not cause any serious histological alteration of the gland; but cytological examination

reveals an increasingly damaged condition of the mitochondria and the Golgi apparatus. These changes seem not to interfere with the life of the cells, however; epithelial culture from the perfused organs yields positive results.

3. The process of cultivation is liable to cause a slight morphological reaction in the thyroid epithelium, which resembles a feeble stimulation of the tissue.

4. Addition of the thyro-stimulating factor from the anterior pituitary to the perfusion fluid causes a stimulation of the cultivated thyroid epithelium which is comparable to, but not so marked as, the corresponding reaction in the living animal.

BIBLIOGRAPHY

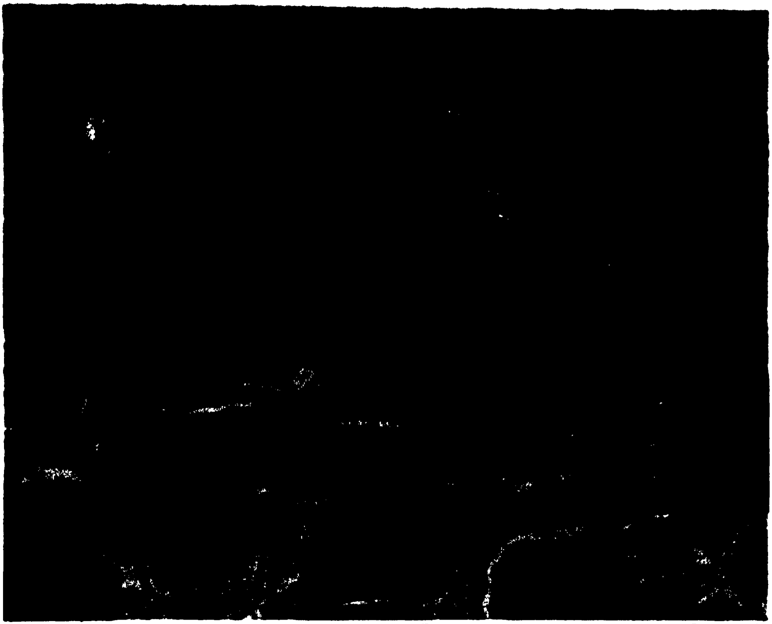
1. Lindbergh, C. A., *J. Exp. Med.*, 1935, **62**, 409.
2. Carrel, A., *J. Exp. Med.*, 1937, **65**, 515.

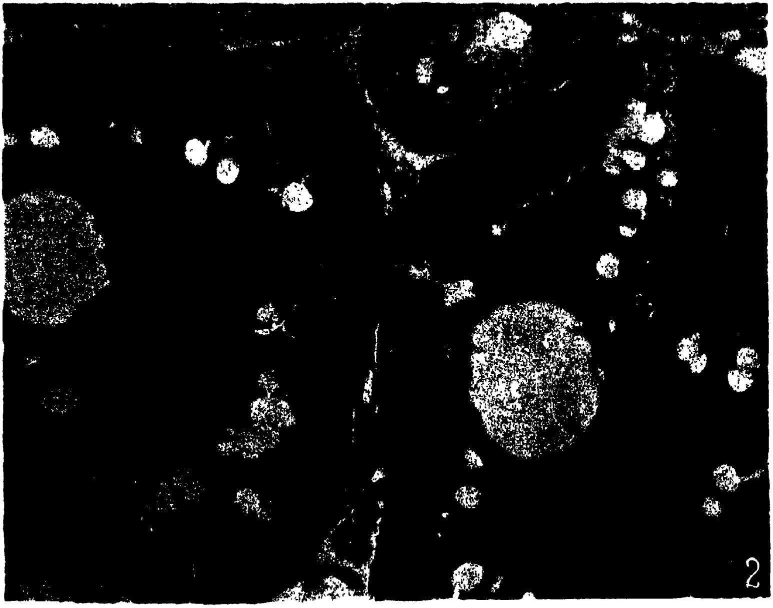
EXPLANATION OF PLATES

PLATE 4

FIG. 1. Left thyroid gland from rabbit (Experiment 428 L), perfused for 48 hours with 40 per cent serum in glucose-Tyrode solution. Plenty of colloid; practically no vacuolization; epithelial cells cuboidal. Fixation: Zenker-formol solution; Heidenhain's iron-hematoxylin stain. × 700.

FIG. 2. Right thyroid gland from same rabbit as in Fig. 1; perfused for 48 hours with 40 per cent serum in glucose-Tyrode solution plus 0.8 per cent thyro-stimulating factor. Vacuolization of colloid; hypertrophic epithelium (note mitotic figure); mitochondria very distinct. Fixation: Zenker-formol solution; Heidenhain's iron-hematoxylin stain. × 700.





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(Okkels: Culture of whole organs. II)

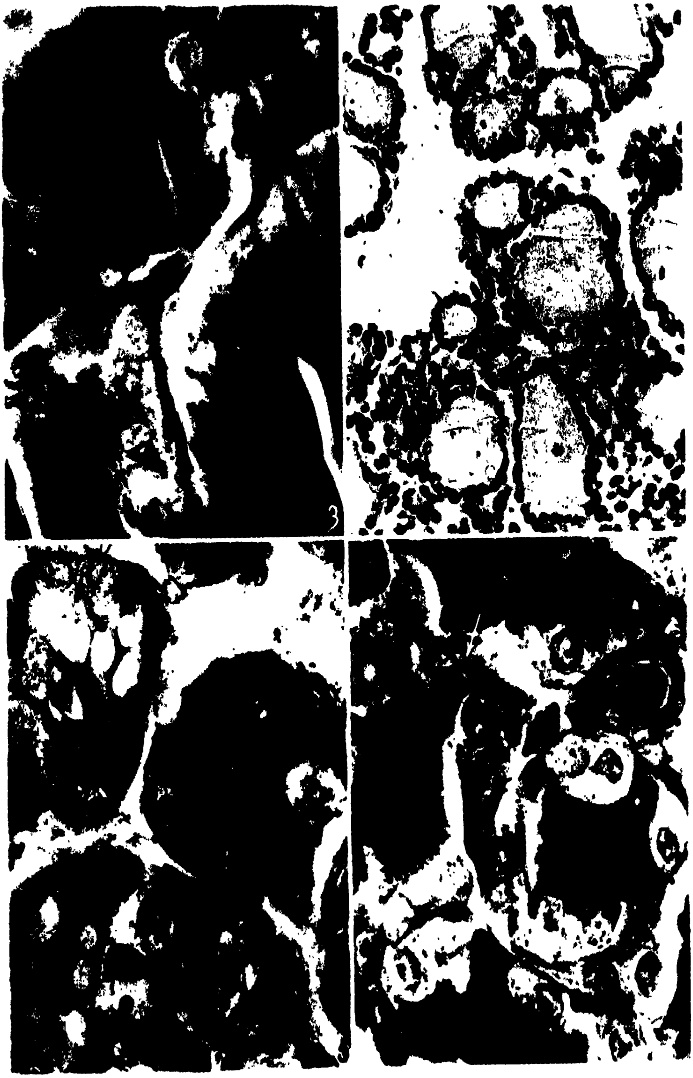
PLATE 5

FIG. 3. Normal shape and size of Golgi apparatus in thyroid cells from a rabbit's gland cultivated for 24 hours in 40 per cent serum plus glucose-Tyrode solution. Osmic saturation (Ludford's modification). × 900.

FIG. 4. Right thyroid gland from cat (Experiment 367 L), perfused for 1 month with 40 per cent serum plus glucose-Tyrode solution. Plenty of colloid; not much vacuolization; flattened epithelial cells. The structure of the left gland, which was not cultivated, was that of a very active thyroid parenchyma, with small vesicles, highly vacuolized colloid and low columnar epithelium. Fixation: Zenker-formol solution; hematoxylin-eosin stain. × 240.

FIG. 5. Vesicles from rabbit thyroid gland cultivated for 24 hours with 0.8 per cent thyro-stimulating factor added to the perfusion fluid. Slight reaction of epithelium; swelling and vacuolization of the supranuclear cytoplasm in a few cells. Fixation: Mann-Kopsch solution; Heidenhain's iron-hematoxylin stain. × 900.

FIG. 6. Vesicles from rabbit thyroid gland cultivated for 24 hours with 0.8 per cent thyro-stimulating factor added to the perfusion fluid. Considerable reaction with many swollen epithelial cells; the mitochondria are distinctly filamentous (note the white arrow). Technique and magnification as in Fig. 5.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THE CULTURE OF WHOLE ORGANS

III. The Problem of Antihormones Studied on Isolated Living Thyroid Glands

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Plates 6 and 7

(Received for publication, June 1, 1937)

Seven years ago a considerable impetus was given to research on the biology of the thyroid gland by the use of extracts from the anterior pituitary. Soon, however, confusion arose on account of the peculiar refractoriness which animals treated with thyro-stimulating pituitary extracts for long periods are liable to develop. Today the study of this refractory state has become a major issue in thyroid biology, as well as in endocrinology generally.

Several investigators have shown that the initial hyperplasia of the thyroid gland produced in animals by the administration of the thyro-stimulating factor from the anterior pituitary may regress and involute to the colloid atrophic state, in spite of continued treatment. Similarly, the metabolic rate, after an initial rise to a maximum on the 7th to the 14th day, falls to normal or below. The mechanism whereby this regression in thyroid activity is brought about is unknown and the published observations and various explanations forwarded by different workers disagree to a very large extent. Some authors have suggested that the refractoriness may be the result of an exhaustion of the thyroid parenchyma. Others assume that specific neutralizing substances are formed and circulate in the blood of the refractory animals.

Collip and Anderson (1) have advanced the so called antihormone theory. According to their view, there may be an antagonistic principle for each hormone which is not the result of an antigen-antibody response but is a true hormone in every way. Collip (2) has lately declared that possibly the antihormone theory should be applied only to the trophic (pituitary) principles. He admits that theoretically a resistant state may be due to a variety of causes, but he stresses the point that the condition is transferable, *i.e.* through serum injections into test animals. Blood serum of guinea pigs which have become resistant to the effects of anterior pituitary extract neutralizes these extracts when injected into normal guinea pigs. Leo Loeb (3), Werner (4) and others were unable, however, to find inhibitory properties in the serum, using the technique of Collip. Werner found that different pituitary preparations, all of them potent thyro-stimulators, have a different ability to induce a refractory state. Katzman, Wade and Doisy (5) were unable to develop a refractory condition in rats by implanting homologous fresh pituitary glands during 9 months. This observation is in keeping with experiments carried out by Krogh and Okkels. Crude pituitary extracts are less liable to cause a state of refractoriness in guinea pigs than are highly purified preparations; further, in long duration experiments an administration of thyroxin to refractory animals is followed at any time by the characteristic rise in metabolism.

Collip and his collaborators claim that refractoriness is bound to develop if only the treatment with the thyro-stimulating factor is continued long enough; and whenever a refractory state has been reached the condition is claimed to be transferable. Scowen and Spence (6) have been able to confirm Collip's observations; at the same time these authors found no evidence of exhaustion of the thyroid cells.

Parkes and Rowlands (7) and others have reported experiments which seem to fit in with the antihormone theory. They found that an antigonadotropic serum, procured by injecting gonadotropic pituitary factor into rabbits over a period of 2 months, will completely inhibit the effect of the specific action of the anterior pituitary extract on the gonads; such serum may be said to effect passive immunization against the secretion of the animal's own pituitary.

The site of formation of the inhibitory principles is unknown. Neither the thyroid nor the hypophysis seems to form inhibitory substances which pass over into the blood. At one time the adrenal cortex was named as a possible source of antihormone during refractoriness. Recently, Gordon, Kleinberg and Charipper (8) have found evidence which points to the spleen as a source of the antigonadotropic principle, results which they interpret as a manifestation of reticulo-endothelial activity.

To our mind, the problems posed by the antihormone theory—especially with regard to thyroid activity—are to a large extent focussed in the two questions:

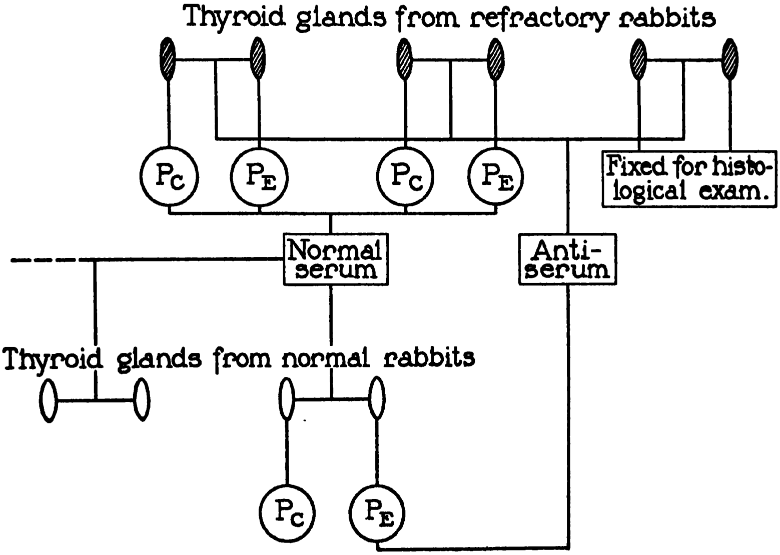
1. Does a "cytological immunity" exist during the state of refractoriness such that the thyroid epithelium itself is unable to respond to any further stimulation by pituitary extracts?

2. Is the serum from refractory animals able to render normal thyroid cells immune to stimulation by anterior pituitary extracts?

In order to answer these questions, we designed the following plan of experiments on isolated thyroid glands, cultivated in the Lindbergh apparatus (9, 10).

Technical

The thyroid glands of refractory rabbits were cultivated in Lindbergh pumps: One gland was perfused in a pump with normal rabbit serum alone (positive control); the other gland was perfused in a pump with normal serum to which had been added thyro-stimulating pituitary factor. The serum from the refractory animals was saved and used in the following way: The thyroid glands of normal rabbits were cultivated in Lindbergh pumps. One gland was perfused in a pump with normal rabbit serum to which thyro-stimulating factor had been added (positive control); the other gland was perfused in a pump with antiserum to which had been added the same amount of thyro-stimulating factor as in the control experiment. The plan of a single set of experiments is shown diagrammatically in Text-fig. 1.


Thyroid glands from refractory rabbits
Pc
PE
Pc
PE
Fixed for histological exam.
Normal serum
Anti-serum
Thyroid glands from normal rabbits
Pc
PE

TEXT-FIG. 1. Diagram showing a single set of experiments. P_E and P_C are Lindbergh pumps with the cultivated experimental and control glands, respectively.

Development of Refractoriness.—Rabbits weighing 2300 to 2700 gm. were used for treatment. It is true that rabbits are much inferior to guinea pigs as far as histophysiological work on the thyroid goes; but the use of guinea pigs was not possible for technical reasons. The high variability of the normal rabbit thyroid, which may be a serious source of error in ordinary experiments (Krogh and Okkels (11)), is, however, sufficiently controlled in the present series because the two thyroid lobes of each animal were always matched as experimental and control specimens.

All the rabbits were maintained on a non-goitrogenic diet. 27 were treated with the thyro-stimulating pituitary factor. The extract was prepared by Ayerst, McKenna and Harrison, according to Collip's directions and standardized to a potency of 50 units per cc. Before the injections were started, we did, however, test different samples of the extract and satisfied ourselves that 1 cc. will bring about a very marked morphological reaction after 1 day in the thyroid of the guinea pig.

Each rabbit was injected with 3 cc. subcutaneously every 2nd day during the 1st week of treatment; then 3 cc. every 3rd day, until 30 cc. had been given. Then the dose was reduced to 1.5 cc. every 4th day during the remaining preparatory period (about 2 months), at the end of which time each animal had received 45 to 50 cc. of the pituitary extract. The weight of the rabbits showed a sharp decline during the first 2 weeks of the treatment; in 22 of the cases the weight increased during the next 2 or 3 weeks until a level was reached which was definitely higher than the initial one (Text-fig. 2); during the last weeks of the experiments the weight of the animals remained practically constant. The histology of the thyroid glands of the 5 animals which failed to develop resistance to the pituitary extract showed the usual hyperplastic state of the parenchyma. The histology of the thyroid glands (unperfused, negative controls; animals bled) from the larger group showed, with the exception of 1 case, a considerable increase in colloid and an atrophic state of the epithelium (Fig. 1). The rate of metabolism was not measured, but we believe that the weight charts, together with the histological findings, can be explained only by assuming that the 22 rabbits had developed refractoriness to the thyro-stimulating factor.

Cultivation.—Several batches of rabbits were prepared for the experiments. As soon as the treatment of one group had been completed, the animals were operated upon. Some of the treated rabbits were etherized and the thyroids dissected for perfusion.[1] Others were etherized and bled to death, the blood being saved in the freezing box overnight to prepare the special culture medium for the normal rabbit thyroid glands (see Text-fig. 1). In all cases the perfusion fluid consisted of 40 per cent rabbit serum diluted with glucose-Tyrode solution (with 5 mg. per cent phenol red as indicator). As a rule the thyroid glands were cultivated only over a period of 24 hours, preliminary study having shown that no sure signs of cytological damage occur during the first 24 to 48 hours of perfusion (Fig. 4). At the same time 24 hours are amply sufficient to permit the morphological effect of the thyro-stimulation to develop.

Histological Methods.—When the circulation was discontinued, the pumps were opened and the glands fixed in Zenker-formol solution and in Ludford's corrosive chloride of mercury-osmic acid, with subsequent osmic saturation. Embedding in paraffin. Serial sections at 4 to 5 microns. Staining in hematoxylin-eosin and Heidenhain's iron-hematoxylin.

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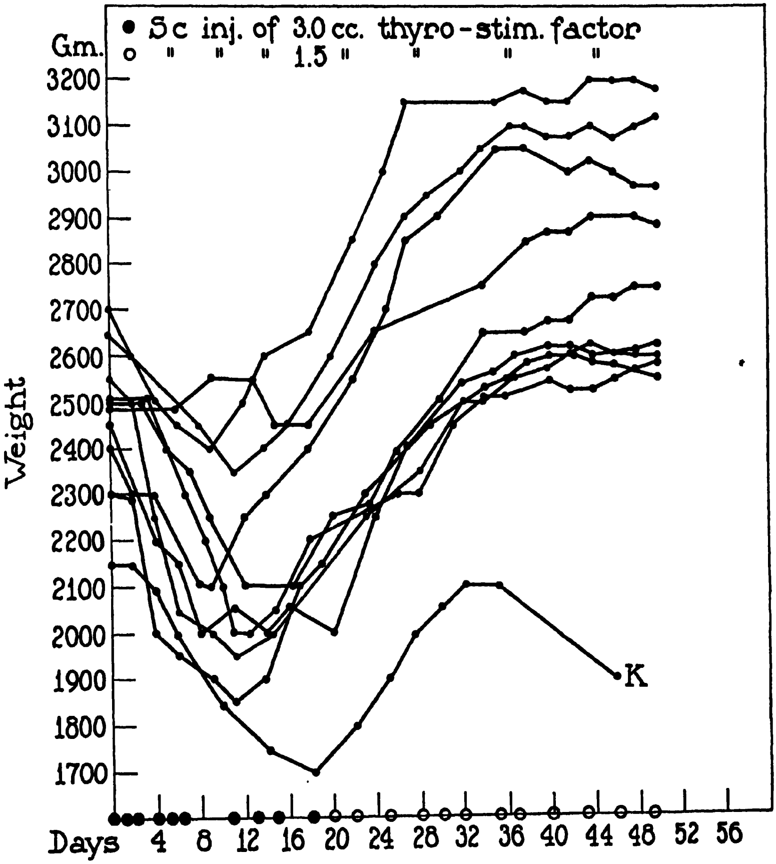
[1] All the animals were operated upon by Dr. Carrel. For details concerning the technique of dissection and the working of the apparatus, the reader is referred to Lindbergh (9) and Carrel (10).



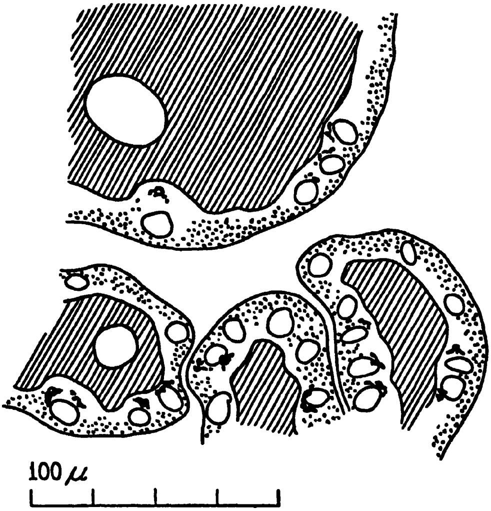
Text-Fig. 2. Chart showing the weights of the last batch of rabbits during 7 weeks of treatment with thyro-stimulating factor. One animal became sick; it had to be killed (K) and consequently was not utilized for any experiment.

Everyone working on the histophysiology of the thyroid gland knows that different parts of the parenchyma may present a different morphological aspect under varying experimental conditions. Usually the central parts of the gland seem to be more active than the periphery. This may be true for untreated animals; it is still more obvious in cases where a certain degree of stimulated condition is induced experimentally. Therefore, it is imperative that the microscopical examination of thyroid glands, such as the ones with which we are concerned in the present series, is carried out with due consideration to this obvious source of error. In our own case, the following estimates as to the degree of secretory activity in the different thyroid glands are based upon observations of topographically corresponding areas of the tissue in matched pairs of experiments. The preparations have been compared again and again, and in many cases several observers have independently examined the same slides. In cases in which degenerated areas of the perfused glands were of considerable extent, new series of sections were made to secure a correct morphological estimate of the functional state of the tissue.

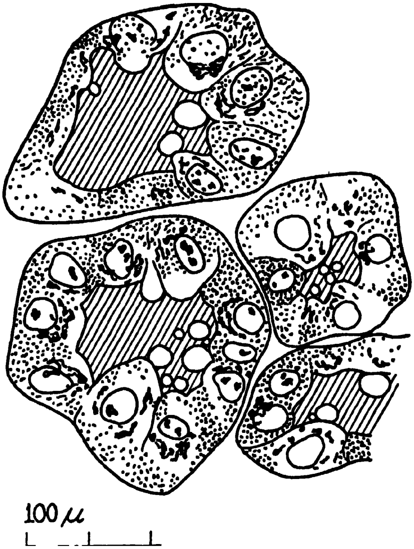
RESULTS

The characteristic histological feature of the thyroid gland during the refractory state is a distention of the vesicles, an accumulation of very dense colloid, and a considerable flattening of the epithelium (Text-fig. 3). Just as the normal thyroid tissue will react to the process of perfusion (12) by developing a slight swelling of the epithelium of the central parts of the gland, so is the thyroid of a refractory animal liable to undergo a slight change during cultivation in the Lindbergh apparatus. There again, it is chiefly the central parts of the gland which are affected; furthermore, the change is only a slight one, causing the thyroid cells to assume a more cuboidal shape. Vacuolization of the colloid is exceptional. If, however, thyro-stimulating pituitary extract is added to the medium, a considerable morphological reaction is observed in the epithelium of the refractory thyroid gland. In several cases this reaction has been comparable to that taking place in normal glands during hormonal stimulation (Text-fig. 4). Only once (Experiment 499) was this effect very slight (Figs. 5 and 6). As a rule the isolated thyroid glands from refractory rabbits responded to the stimulating effect of anterior pituitary extract by a definite swelling of epithelial cells over large areas of the parenchyma, by enlargement of the Golgi apparatus and by an extensive vacuolization of the colloid (Figs. 7 and 8).

In the experiments in which the possible neutralizing effect of the serum from refractory animals was tested on isolated normal thyroid glands, the observations were less consistent. In 3 of the experiments, we found practically the same degree of hypertrophy and increased cellular activity in both lobes of the thyroid (Figs. 11 and 12). In 1



Text-Fig. 3. In spite of the fact that the thyroid parenchyma is atrophic during the condition of refractoriness, the Golgi apparatus is quite distinct and easily impregnable with osmic acid.



Text-Fig. 4. When the thyroid epithelium of glands from refractory rabbits responds to pituitary stimulation, the swelling of the cells and the increase of the Golgi apparatus may be considerable (compare with Text-fig. 3).

TABLE I

Response of Perfused Rabbit Thyroid Glands to Stimulation by Anterior Pituitary Extract

No.	Negative controls	Positive controls	Experiments	Amount anterior pituitary extract	Remarks
Thyroid Glands from Refractory Rabbits					
				cc.	
486		0			
487			+	2	
488		+			
489			++	2	
496		(+)			Perfused for 22 hrs.
497	0				Not cultivated
498		0			Epithelium very atrophic
499			+ or 0	2	
525	+				Not cultivated
526			++	4	2 cc. anterior pituitary extract for 22 hrs. + 2 cc. for 5 hrs. Total: 4 cc. for 27 hrs.
531		+			
532			++	2	
533		0			
534			+	2	
540		+			
541			++	2	
542		0			
543			++	2	

0 indicates a morphologically inactive tissue of the colloid type.

+ to +++ indicate varying degrees of morphological activity.

The concentration of serum was 40 per cent in all cases. The amount of perfusion fluid 250 cc.

Where nothing special is indicated in the last column of the table the duration of the experiment was 24 hours.

TABLE I—*Concluded*

No.	Negative controls	Positive controls	Experiments	Amount anterior pituitary extract	Remarks
Normal Thyroid Glands Perfused with Serum from Refractory Rabbits					
				cc.	
490		+++		2	Considerable degeneration
491			+	2	
494		+++		2	Considerable degeneration
495			+++	2	
500		++		2	
501			+	2	
527		+++		1	1 cc. anterior pituitary extract for 3 hrs.
528			++	1	1 cc. anterior pituitary extract for 24 hrs.
535		++		2	
536			++	2	
546		+++		2	
547			+++	2	

case (No. 528) the morphology of the lobe perfused by antiserum showed a less active state than the corresponding control. In the remaining 2 experiments of the group (Nos. 491 and 501), the controls were definitely more active morphologically than the glands perfused with antiserum; probably, therefore, an inhibitory effect had been achieved here (Figs. 9 and 10).

All the positive controls in this experimental group showed distinct histological signs of considerable secretory activity, as would be expected in cases in which normal thyroid glands perfused with normal serum were exposed to the stimulating effect of a potent extract from the anterior pituitary.

The degree of the morphological reactions observed in all the perfused glands is indicated in Table I, where both groups of experiments and the results are recorded.

DISCUSSION

The histological examination of perfused and stimulated thyroid glands from refractory rabbits shows that the thyroid epithelium itself is not unable to respond to the stimulation by pituitary extract during the state of refractoriness in the animal. We must, therefore, answer in the negative the first question put in the introduction of this paper: There is no evidence for the existence of a cytological immunity of the thyroid epithelium in rabbits which have been rendered immune to the action of the thyro-stimulating factor from the hypophysis. The thyroid epithelium may be very atrophic—exhausted—during the state of refractoriness. But the thyroid cells of the isolated gland can be made to respond to pituitary stimulation within 24 hours when cultivated.

The second question, whether the serum from refractory rabbits is able to antagonize the effect of the thyro-stimulating factor from the hypophysis, is only partly answered by our results. In 3 out of 6 cases, no sure signs of any antagonism between the antiserum and the pituitary extract were observed. It would seem as if the antiserum in the remaining 3 cases had inhibited the action of the thyro-stimulating factor upon normal thyroid cells. But the serum did not neutralize the effect of the pituitary extract completely. And the degree of inhibition was surprisingly small, considering the large amount of serum utilized in each experiment.

In view of the fact that the thyroid cells themselves, as the first group of experiments show, are not fundamentally changed by the state of refractoriness as concerns their ability to react morphologically upon pituitary thyro-stimulation, it seems logical to assume either that the inhibitory effect of the antiserum is a simple quantitative matter of neutralization, or that an extra-thyroid mechanism is involved. The first assumption is hardly tenable. If we suppose that inhibition is determined simply by the amounts of antagonistic principles—the antihormone and the pituitary extract—actually present as a mixture in the perfusion fluid, it is hard to explain why similar experiments in living animals imply admixture of only a very much smaller amount of antiserum to obtain a complete neutralization. It should be remembered that in each of our experiments at least 80 cc. of serum from refractory animals were made to react upon one single lobe of thyroid gland in an attempt to counteract the effect of 1 or 2 cc. of an anterior pituitary extract. Scowen and Spence (13) have been able to prevent the occurrence of thyroid hyperplasia in guinea pigs by injecting subcutaneously 2 cc. of antiserum against 1 cc. of an active anterior pituitary extract given intraperitoneally. Similar results were obtained in rabbits. These investigators believe that the neutralizing substance is a hormone and not an antibody formed against a foreign protein.

All the same, in the experiments on the isolated thyroid gland, and with the use of much greater amounts of serum, it is possible to obtain only a comparatively small inhibitory effect, or none at all. Probably, therefore, organs or tissues other than the thyroid epithelium are involved in the mechanism underlying the phenomenon of refractoriness to the thyro-stimulating factor. It should be noted in this connection that the isolated, perfused thyroid glands cannot always be considered completely deprived of their nerve supply from sympathetic ganglia; and nerve cells have been observed to remain histologically normal during perfusion in the Lindbergh apparatus for a considerable length of time. Very little is known concerning the interrelationship between the nervous system and the thyroid, but living nerve tissue may actually be present in some of the perfused thyroid preparations. Whether this is of any significance as far as our particular problem goes, it is impossible to decide at present.

SUMMARY

1. Perfusion of isolated thyroid glands from rabbits, made refractory to the action of the thyro-stimulating factor from the hypophysis by repeated injections for 2 months, has shown that in this condition the thyroid epithelium responds to pituitary stimulation.

2. The serum from refractory rabbits does not neutralize completely the thyro-stimulating effect of anterior pituitary extract upon an isolated normal thyroid gland.

3. It is believed that tissues other than the thyroid play a rôle in bringing about the phenomena which have been described as refractoriness against a prolonged action of the thyro-stimulating pituitary factor.

BIBLIOGRAPHY

1. Collip, J. B., and Anderson, E. M., *J. Physiol.*, 1934, **82**, 11.
2. Collip, J. B., *Ann. Int. Med.*, 1935, **9**, 150.
3. Loeb, L., *Science*, 1934, **80**, 252.
4. Werner, S. C., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 390.
5. Katzman, P. A., Wade, N. J., and Doisy, E. A., *Endocrinology*, 1937, **21**, 1.
6. Scowen, E. F., and Spence, A. W., *Brit. Med. J.*, 1934, **2**, 805.
7. Parkes, A. S., and Rowlands, I. W., *J. Physiol.*, 1936, **88**, 305.
8. Gordon, A. S., Kleinberg, W., and Charipper, H. A., *Anat. Rec.*, 1937, **67**, No. 4, suppl. 3, 19.
9. Lindbergh, C. A., *J. Exp. Med.*, 1935, **62**, 409.
10. Carrel, A., *J. Exp. Med.*, 1937, **65**, 515.
11. Krogh, M., and Okkels, H., *Klin. Woch.*, 1936, **15**, 203.
12. Okkels, H., *J. Exp. Med.*, 1937, **66**, 297.
13. Scowen, E. F., and Spence, A. W., *J. Physiol.*, 1936, **86**, 109.

EXPLANATION OF PLATES

PLATE 6

FIG. 1. Uncultivated thyroid gland from refractory rabbit. Abundant colloid; practically no vacuolization; very atrophic epithelium. Fixation: Zenker-formol solution. Heidenhain's iron hematoxylin stain. × 650.

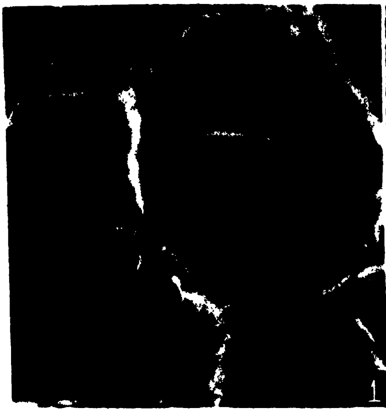
FIG. 2. Histology of normal rabbit thyroid gland (Experiment 428 L; colloid type) for comparison with Fig. 1. Same technique and magnification.

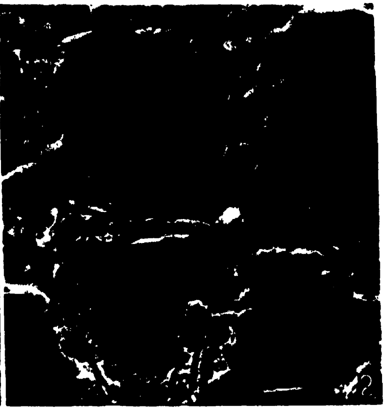
FIG. 3. Right lobe of uncultivated thyroid gland from rabbit (Experiment 497), showing atrophy of the epithelium during refractoriness. Same technique and magnification as Fig. 1.

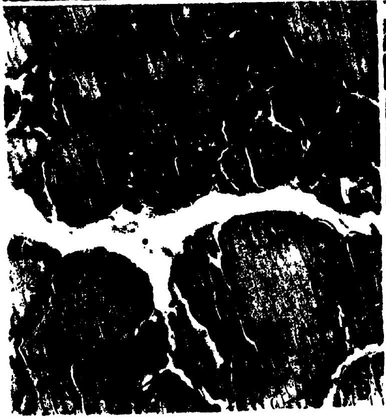
FIG. 4. Left lobe of the thyroid gland from the same rabbit as in Fig. 3 (Experiment 496 L), cultivated for 22 hours with 40 per cent serum plus glucose-Tyrode solution. The size of the epithelial cells is somewhat increased but there is no vacuolization. Same technique and magnification as Fig. 1.

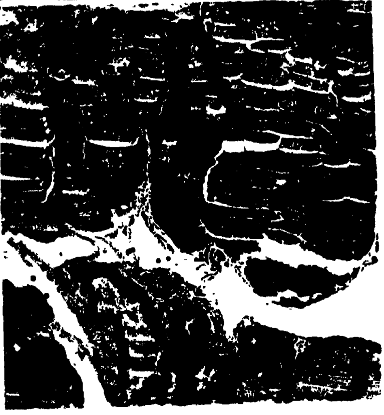
FIG. 5. Experiment 498 L. Atrophic thyroid gland from a refractory rabbit. Duration of cultivation, 24 hours. The cells are flattened and the colloid is not vacuolated. Fixation: Zenker-formol solution; hematoxylin-eosin. × 195.

FIG. 6. Experiment 499 L. Other lobe of thyroid gland from same animal as in Fig. 5. Duration of cultivation: 24 hours; 2 cc. of thyro-stimulating factor was added to the perfusion fluid. Example of very slight stimulation (compare with Table I). The general histological aspect of the gland is the same as in the control, but there is a definite increase of the fine vacuoles in the colloid. Histological technique and magnification as Fig. 5.









(Okkels: Culture of whole organs. III)

PLATE 7

FIG. 7. Experiment 542 L. Histologically inactive thyroid gland from refractory rabbit. Same technique and magnification as Fig. 5.

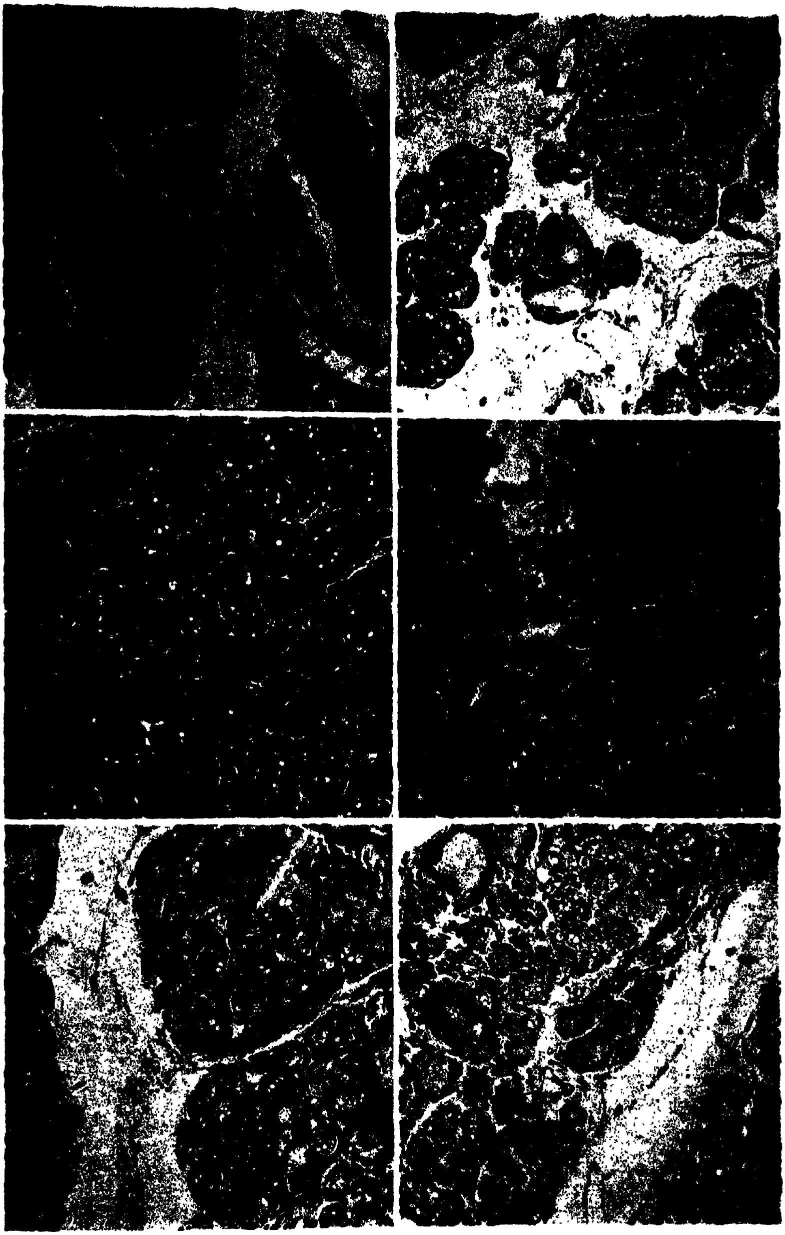
FIG. 8. Experiment 543 L. Other lobe of thyroid gland from same animal as in Fig. 7. Medium degree of response to stimulation by anterior pituitary extract. Same technique and magnification as Fig. 5.

FIG. 9. Experiment 490 L. Hyperplastic thyroid gland from normal rabbit. Typical effect of thyro-stimulating pituitary factor. Same technique and magnification as Fig. 5.

FIG. 10. Experiment 491 L (perfused with antiserum). Other lobe of thyroid gland from same animal as in Fig. 9. The effect of the thyro-stimulating factor is very considerably inhibited. Example of maximum effect of the antiserum; compare with Table I. Same technique and magnification as Fig. 5.

FIG. 11. Experiment 546 L. Hyperplastic thyroid gland from normal rabbit. Typical effect of thyro-stimulating pituitary factor. Same technique and magnification as Fig. 5.

FIG. 12. Experiment 547 L (perfused with antiserum). Other lobe of thyroid gland from same animal as in Fig. 11. The tissue shows about the same degree of hyperplasia and stimulation. Same technique and magnification as Fig. 5.



(Okkels: Culture of whole organs. III)

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STUDIES ON THE SENSITIZATION OF ANIMALS WITH SIMPLE CHEMICAL COMPOUNDS

IV. ANAPHYLAXIS INDUCED BY PICRYL CHLORIDE AND 2:4 DINITROCHLOROBENZENE

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In previous communications (1–4), report has been made of sensitization effects in guinea pigs following intracutaneous or superficial administration of a number of simple chemical compounds, *e.g.*, nitrosodimethylaniline and 2:4 dinitrochlorobenzene. Thus, animals treated intradermally with small quantities of the substances exhibit distinctly increased reactions to subsequent intradermal injections, and give erythematous reactions when a solution of the incitant is spread on the skin. A study of various nitrochlorobenzenes then demonstrated a parallelism between skin sensitizing capacity and chemical reactivity, consistent with the idea that sensitization effects are due to conjugated antigens formed *in vivo*. That artificially conjugated antigens—azoproteins—can sensitize (anaphylactically) to the conjugate, the reactions being specific for the substance linked to protein, had been shown previously (5; *cf.* 6), but there was no direct evidence that sensitivity to simple substances may depend upon the formation of such antigens (7). Another result was that in the case of acyl chlorides, which also are able to sensitize, skin sensitiveness and anaphylaxis were produced simultaneously upon repeated intracutaneous injections, indicating a relationship in the causation of the two effects. In the present paper, this question is further investigated.

EXPERIMENTAL

Anaphylaxis Experiments with Picryl Chloride and 2:4 Dinitrochlorobenzene.—In the experiments cited above, benzoyl chloride and *p*-chlorobenzoyl chloride gave rise to both skin sensitization and anaphylaxis. The formation in these instances of conjugate antigens evidently responsible for the production of anaphylaxis is not particularly surprising, since the substances are easily decomposed by water and are highly reactive, *e.g.* with proteins. In this respect the experiments are somewhat similar to the sensitizations obtained with diazonium and diazoamino compounds (8, 9). It seemed necessary to make investigations on the possibility of producing anaphylaxis with substances that are relatively stable and are known to cause allergic phenomena in human subjects. Two substances were chosen for study, picryl chloride, previously shown to sensitize guinea pigs (1) and capable, moreover, of producing sensitization in human beings upon intracutaneous administration,[1] and 2:4 dinitrochlorobenzene, which is known to cause contact dermatitis in a large number of industrial workers handling it (10–12). Picryl chloride, although combining readily with proteins in alkaline solution, is not decomposed by water at room temperature for a considerable period of time, and can be recrystallized from boiling alcohol; it does, however, react slowly with serum proteins at serum alkalinity. The second substance, 2:4 dinitrochlorobenzene, is stable in boiling water for hours and does not combine with serum proteins to any appreciable extent without the addition of alkali.

Guinea pigs were sensitized by repeated intracutaneous administration on the dorsum, the course commonly consisting of about fifteen daily injections, six a week, each of 1/400 mg. picryl chloride in 0.1 cc. saline.[2] The commercial preparation was used after two recrystallizations from a benzene-alcohol mixture, m.p. 82° (uncorrected). For the injections, solutions of the requisite concentration were prepared by diluting in saline an alcoholic 0.3 per cent solution.

White male guinea pigs weighing 350–450 gm., mostly albinos, were used for the sensitizations. The development and the degree of skin sensitivity to picryl chloride correspond in a general way to observations reported with 2:4 dinitrochlorobenzene (1). With daily injections, the reactions being recorded 24 hours later, evidence of a heightened response would usually be noted between 6 and 8 days after the first injection, the sensitivity gradually developing to give elevated, pink areas 10 to 15 mm. in diameter, often with blanched or livid centers, at times with a necrotic spot. A period of 2 or 3 weeks was allowed between the last injection of the course and the final testing, which was made by spreading on the belly, after clipping the hair, 1 drop of a 2 per cent solution of picryl chloride in olive oil; for subsequent tests, new belly sites were chosen each time. The reactions were read 18 to 24 hours later, following use of a depilatory 2 or 3 hours before. On normal animals, similarly treated as controls, reactions were faint or absent. Of 134 pigs treated with picryl chloride by the above or a comparable method, only seven did not show evidence of sensitization; this uniformity closely resembles experiences with 2:4 dinitrochlorobenzene (4).

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[1] Personal communication from Dr. Marion B. Sulzberger.

[2] Lately it has been found that very satisfactory skin sensitization can be attained by intracutaneous injection of larger quantities of picryl chloride than those used before, given simultaneously in several sites (1/50 mg. injected in each of 7 sites, test made 1 month later).

For the preparation of protein conjugates 1 millimol of picryl chloride in 5 cc. chloroform was shaken vigorously for 15 minutes with a mixture of 30 cc. horse serum (or guinea pig serum) and 15 cc. N/2 Na_2CO_3, the temperature being kept at about 5°C. After the removal of insoluble material by centrifugation in the cold, the supernatant was acidified to maximum precipitation, and the picryl protein sedimented by spinning. The protein compound was dissolved in water with addition of alkali to pH 8–9, the reaction adjusted close to neutrality, and a small amount of insoluble material centrifuged off. For purification, the protein was precipitated by treatment with 5 volumes of alcohol, centrifuged, and redissolved in water at slight alkalinity; it was finally precipitated with acid, washed once with saline by centrifugation, and dissolved in saline at slight alkalinity. The solution was made approximately neutral, becoming somewhat turbid thereby, and the concentration (about 2 to 3 per cent) was determined.

Recently we have found that the protein compounds can be made by gentle shaking of serum with finely ground picryl chloride; this method can also be used with 2:4 dinitrofluorobenzene (see below).

With intracutaneous injections of picryl chloride, as previously found with *p*-chlorobenzoyl chloride (2), guinea pigs were found to develop anaphylactic, in addition to skin, sensitivity. As seen in Table I, typical anaphylactic shock resulted when picryl protein conjugates were given intravenously. While with the batch of animals shown in the table 4 mg. represented approximately the limiting quantity of antigen to produce fatal shock, this value fluctuated with different series of sensitized animals. It was as low as 1/5 mg. in a group which had received six weekly intradermal injections of picryl chloride (tested 10 days later with "picryl horse serum"), and also in a lot given ten to fourteen daily injections (test made with "picryl guinea pig serum" after 4 weeks' rest); in other experiments, only a small proportion of the animals were fatally shocked with 10 or 20 mg. doses of antigen.

As a complication, in some cases it seemed that large doses were less effective than smaller ones in demonstrating anaphylaxis (*cf.* 8). The interval between the last sensitizing injection and the intravenous injection of picryl protein may be as short as a week; in one experiment in which animals were tested after intervals of 1 week and 5 weeks, the degree of anaphylactic sensitivity had diminished by the 5th week.

TABLE I

Anaphylaxis in a group of guinea pigs given daily intradermal injections of 1/400 mg. picryl chloride for 15 days, and injected intravenously with picryl protein between 8 and 12 days after the last skin injection. Figures in parentheses indicate change in temperature (°C.).

No.	Amount injected	Intravenous injection of picryl horse serum
	mg.	
1	12	Slight symptoms (−1.1)
2	10	Typical anaphylaxis † 4 min.*
3	8	" " † 6 "
4	8	" " † 13 "
5	8	" " † 4 "
6	8	Severe shock, recovered (−5.9)
7	4	Typical anaphylaxis † 4 minutes
8	4	" " † 6 "
9	4	Slight symptoms (−1.2)
10	2	Coughs, eyes running, labored breathing (−1.5)
11	1	Typical anaphylaxis † 7 min.
12	1	Slight symptoms (−1.0)
13	1/5	No symptoms (+0.2)
		Controls
14	16	No symptoms (−0.6)
15	16	" " (−0.2)
16	8	" " (−0.4)
17	8	" " (−0.4)
18	4	" " (−0.6)

* The symbol † signifies death of animal; in all cases the autopsy findings have been characteristic.

The presence of antibodies in the serum of guinea pigs sensitized by intradermal injections of picryl chloride was demonstrable by passive transfer, using the Schultz-Dale method (see 13).

The sensitized animals were bled between the 6th and the 14th day after the final intradermal injection of picryl chloride, and the serum, commonly in 3 cc. amounts, was injected intraperitoneally into virgin female guinea pigs weighing 180–250 gm. After 24 or 48 hours the animals were exsanguinated by heart puncture and the excised uterine horns were rinsed and mounted in a Dale apparatus in separate chambers of 20 cc. capacity, the recording being made at about threefold magnification. The oxygenated bath usually was the fluid recommended by Dale (13) (NaCl 0.9 per cent, KCl 0.042 per cent, $CaCl_2$ 0.012 per cent, $NaHCO_3$ 0.05 per cent, in glass-distilled water); infrequently the amount of calcium chloride was reduced to one-half. The antigen (1 or 2 mg., or less, of picryl protein made with horse serum) was added to the bath in a volume of 0.2 cc. or less, the resultant concentration being without effect upon the uterine horns of normal guinea pigs. If a contraction ensued, and specific desensitization was to be demonstrated, the horn was allowed to relax fully and was then washed by repeated changes of oxygenated fluid; thereupon the same dose of antigen which had caused the first contraction was added to the bath. Finally, to test the condition of the muscle, an addition of histamine was made.

A representative experiment of this sort is given in Text-fig. 1, where it is seen that the passively sensitized muscle responded to picryl protein even at 1:80,000 dilution and that when the test was repeated the uterus was seen to be desensitized. Anaphylactic antibodies were found with most batches in a large proportion of animals sensitized by intradermal injections of picryl chloride as described above, *e.g.*, with one group, eight out of ten guinea pigs, and in another experiment seven out of nine, gave positive transfers; in some lots definitely poorer effects were obtained. On the whole, these results would seem to compare not unfavorably with those obtained in homologous passive transfer experiments with sera from animals injected with proteins (14, 15).

Many of the transfers effected with 3 cc. amounts of serum resulted in maximal contractions maintained 2 to 6 minutes when the horns were exposed to picryl horse serum in the usual concentration of 1:20,000. With the most active sera, 1 cc. was adequate for sensitization. In some instances where uteri appeared to be sensitized highly, the antigen was employed in greater dilutions; in one case, there was a maximal sustained contraction to an antigen concentration of 1:2,000,000, which indicates a degree of sensitivity close to that shown by highly sensitive uteri as a result of customary protein immunization (*cf.* 13). It may be mentioned that there seemed to be some parallelism between the intensity of skin reactions elicited by the later preparatory injections and the content of anaphylactic antibodies in the serum; in some series of animals, however, such a relationship was not seen in the degrees of anaphylactic and cutaneous sensitivities, the latter tested by applying oil solutions on the skin several weeks after the last treatment.

Precipitins were detected in some of the sera which were among the most active in conferring passive sensitization, picryl protein being used as antigen in ring tests. The rings, although faint and detectable only with clear sera, were definite; the optimal concentration of picryl guinea pig serum was usually 1:30,000. Attempts to transfer skin sensitiveness passively by means of sera containing anaphylactic antibodies and precipitins have so far been negative.

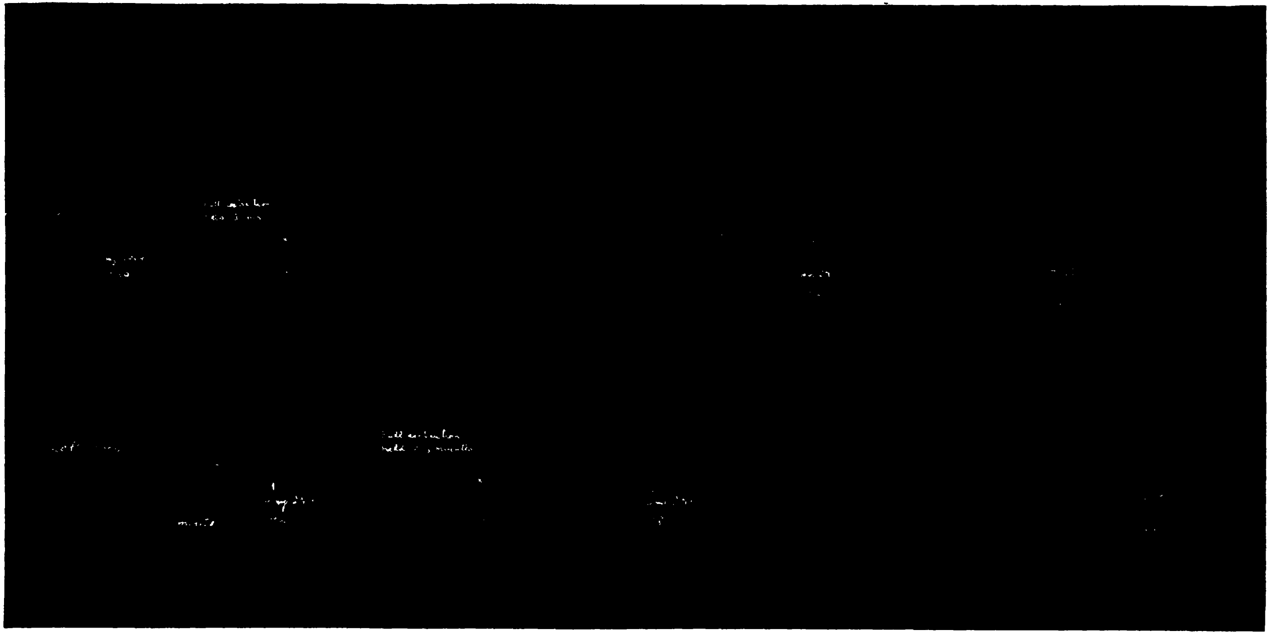
While in the above experiments skin sensitization and anaphylactic antibodies were developed by intradermal injections as stated, it is of interest that in a preliminary experiment anaphylactic antibodies were found also in guinea pigs sensitized by continued daily application of one drop of a 2 per cent solution of picryl chloride in olive oil to the intact skin, anaphylactic sensitivity arising in a normal guinea pig from the intraperitoneal injection of 7.5 cc. pooled serum taken from three cutaneously sensitized guinea pigs.

Analogous experiments were carried out on guinea pigs sensitized to 2:4 dinitrochlorobenzene by means of repeated intracutaneous injections, as in the case of picryl chloride.

The development of skin sensitivity to this substance has been described (1); cutaneous sensitivity was tested by superficial application of a 1 per cent solution in olive oil. On account of its greater reactivity as compared with the chloro compound, 2:4 dinitrofluorobenzene (16) was used in the preparation of 2:4 dinitrophenyl protein conjugates. The latter were prepared with guinea pig or horse serum, the method for picryl protein being followed in the main. The guinea pig serum preparation was made by shaking in the cold for 10 minutes; in reprecipitating the protein derivative, careful adjustment of the pH and addition of salt were necessary; the antigen was used after dialysis against isotonic saline. The horse serum preparation was obtained in a similar manner. The solution tended to become turbid when kept in the ice box at neutral reaction, and before use the amount required was considerably clarified by cautious addition of NaOH and then adjusted with HCl until almost neutral.

In Schultz-Dale experiments with actively sensitized guinea pigs, since the animals usually were rather heavy by the time of testing, and the horns showed

TEXT-FIG. 1. Anaphylactic antibodies in serum of guinea pig given fifteen daily intradermal injections of picryl chloride (see text), 7 days after the last injection, demonstrated by passive transfer. Recipient guinea pig (235 gm.) was injected intraperitoneally with 3 cc. serum, and the uterine horns were tested 2 days later. Additions of picryl horse serum (P.H.) to the bath (20 cc. Dale solution) were made at the times noted after mounting the horns; the sign X designates washing out of the chamber; histamine dihydrochloride (Hist.) was finally added (concentration 1:5,000,000 of the free base).



TEXT-FIG. 2. Anaphylactic response in a guinea pig sensitized by fifteen daily intradermal injections of 2:4 dinitrochlorobenzene (see text) and tested by Dale's method with 2:4 dinitrophenyl horse serum (D.N.H.) as antigen. 11 days after the last injection, the uterine horns of the animal (360 gm. body weight) were mounted in 20 cc. modified Dale solution (calcium chloride reduced to one-half), and the antigen was added as shown. Histamine (Hist.) was used in a concentration of 1:10,000,000.

spontaneous contractions in the above mentioned Dale solution, modified bath fluids were employed, either the Dale solution with the amount of calcium chloride reduced to one-half or one-quarter (animals weighing 325–400 gm.) or the calcium-free solution used by Bristol and Fleischner (17). For the latter (see 18), a stock solution is made with 10.5 gm. NaCl, 0.5 gm. KCl, 0.1 gm. $MgCl_2$, 5 cc. N H_3PO_4, 50 cc. water, and the bath fluid is prepared from 50 cc. stock solution, 5 cc. N Na_2CO_3, and 1000 cc. water. Along with repression of spontaneous contraction in these solutions, the specific reactions are apparently diminished and the tests less sensitive.

TABLE II

Anaphylactic response of a group of guinea pigs given 6 weekly intradermal injections of 1/400 mg. 2:4 dinitrochlorobenzene, 2:4 "dinitrophenyl guinea pig" serum being injected intravenously 4 weeks after the last skin injection.

No.	Amount injected	Intravenous injection of 2:4 dinitrophenyl protein
	mg.	
19	20	Severe symptoms (chronic type), recovered
20	20	Typical anaphylaxis, † 4 min.
21	20	No symptoms
22	20	Typical anaphylaxis, † 4 min.
23	10	Questionable symptoms
		Controls
24	20	No symptoms
25	20	" "

Guinea pigs sensitized by intracutaneous injections in the manner stated were tested after 7 to 40 days by intravenous injection with the protein conjugate. Resultant anaphylactic symptoms were variable, and the instances of fatal shock were few. Nevertheless, as seen in Table II (which presents the best result thus far obtained), the fact that the intracutaneous administration of 2:4 dinitrochlorobenzene can induce an anaphylactic state could be demonstrated beyond doubt. It would seem probable that continued study should determine experimental conditions under which the results will be more regular and passive transfer can be demonstrated.

Further evidence was forthcoming from experiments made with the Schultz-Dale method, the horns of sensitized guinea pigs being tested with 1:10,000 or 1:20,000 dilutions of 2:4 dinitrophenyl pro-

TABLE III

Anaphylactic desensitization by subcutaneous injection of picryl protein in two batches (A and B) of guinea pigs sensitized by 15 daily intradermal injections of 1/400 mg. picryl chloride. 12 days after the last skin injection, part of the animals were given 10 mg. picryl guinea pig serum subcutaneously; next day, all were tested for skin sensitiveness, and one day later picryl horse serum was injected intravenously. Figures in parentheses indicate change in temperature (°C.). The skin reactions on normal animals ranged from negative to faint pink.

Animals given desensitizing injection				Sensitized controls			
No.	Application of a 2 per cent solution of picryl chloride in olive oil on the skin	Intravenous injection of picryl horse serum		No.	Application of a 2 per cent solution of picryl chloride in olive oil on the skin	Intravenous injection of picryl horse serum	
		Amount	Symptoms			Amount	Symptoms
		mg.				*mg.*	
			Group A				
26	p., el.*	10	None**	30	dp., m.el.	10	† 4 min.
27	p., el.	10	None**	31	p., sl.swol.	10	† 11 min.
28	pp., sl.el.	10	None (+0.8)	32	pp.–p., el.	10	† 4 min.
29	pp.–p., sl.el.	5	None (−0.9)	33	p., el.	5	Moderate to severe (−0.7)
				34	pp., sl.el.	10	Moderate (−1.7)
				35	pp., sl.el.	10	Moderate (−1.9)
				36	pp.–p., sl.el.	10	† 30 min.
			Group B				
37	p., sl.el.	10	None (−0.1)	40	pp., sl.el.	10	Slight to moderate (−1.8)
38	p., sl.el.	10	None (+0.5)	41	p., sl.el.	10	† 14 min.
39	p., el.	10	None (−1.0)	42	p., sl.el.	10	Moderate (−2.1)

*The following abbreviations are used: faint pink (fp.), pale pink (pp.), pink (p.), dark pink (dp.), slightly elevated (sl.el.), elevated (el.), markedly elevated (m.el.), swollen (swol.), blanched center (bl.c.).

**Temperature change not determined.

tein compounds prepared from horse or guinea pig serum. Here again the results were inconstant, and negative with two small batches of animals, which incidentally did not exhibit a high degree of skin sensitivity; in other lots of guinea pigs, however, definitely positive reactions occurred, *viz.* with two out of eight, and in five out of a group of fifteen (tested 9 days after the last intradermal injection), and in four out of nine (examined after a rest of 32 to 50 days). The reactions ranged from relatively weak contractions to, in the majority of cases, maximal contractions sustained for 1 to 3 minutes, specific desensitization being demonstrated regularly; such a record is shown in Text-fig. 2.

TABLE IV

Failure of subcutaneously administered picryl protein to desensitize the hypersensitive skin of selected guinea pigs previously given intradermal injections of picryl chloride. After the first skin test made by applying 1 drop of a 2 per cent solution of picryl chloride in olive oil to the skin of the belly, half of the animals were reserved for comparison, the others were injected subcutaneously with picryl-guinea pig serum (10 mg. on the 2nd, 4th, and 8th days, 20 mg. on the 10th day), and a second skin test was made in the same way on the 12th day.

Animals given desensitizing injections			Sensitized controls		
No.	First skin test	Second skin test (after four injections of picryl protein)	No.	First skin test	Second skin test
43	p., bl.c., swol.	dp., m.el.	47	p., el.	pp.
44	pp., sl.el.	pp.	48	pp., sl.el.	pp.
45	pp.-p., sl.swol.*		49	pp.-p., sl.swol.	p., swol.
46	p.	p.	50	pp.	pp.-p.

* Animal died within a few hours after the first subcutaneous injection of picryl protein.

Desensitization Experiments with Picryl Protein.—With animals sensitized to picryl chloride, experiments aimed at desensitization were carried out by administering picryl guinea pig serum subcutaneously prior to the intravenous shocking injection. The subcutaneous injections were not seldom followed by local reactions, consisting of edema, more pronounced than in normal animals, and sometimes reddening of the skin. The results as presented in Table III show that anaphylactic desensitization could be achieved regularly. In contrast to this, it will be seen that the reactivity of the skin to superficial application of picryl chloride was not concomitantly abolished, and indeed even repeated subcutaneous injections of picryl protein had no noticeable influence on the degree of skin sensitiveness (Table IV). While we have not investigated the subject particularly, in an experiment with a few animals we were able to desensitize the skin by long continued daily applications of an olive oil solution of the incitant, 2:4 dinitrochlorobenzene. In a similar way Kobayashi (19), working with guinea pigs sensitized with an extract of *Rhus vernicifera,* reported desensitization by long continued painting with the extracts. By means of subcutaneous injections in human beings, Blank and Coca (20) found that a certain degree of immunity to contact with poison ivy develops.

COMMENT

The above experiments demonstrate that certain simple substances which cause human hypersensitiveness, with skin manifestations, produce upon intracutaneous injection into guinea pigs both skin sensitivity and anaphylactic sensitization. This has been shown with picryl chloride and with 2:4 dinitrochlorobenzene, which are capable of inducing cutaneous sensitization in human beings. It is noteworthy, indeed, that in these cases very small quantities of simple compounds can produce anaphylactic sensitization, evidently through combination with some substance of the animal body.

From our results, it appears that although both the compounds mentioned sensitize guinea pig skin in like manner they probably differ quantitatively in their capacity to evoke an anaphylactic state. While this distinction is one of degree only, for also with 2:4 dinitrochlorobenzene unquestionable anaphylactic effects were obtained, nevertheless the result taken in conjunction with the skin effects would indicate differences in the mode of anaphylactic sensitization and sensitization of the skin to superficial application of the incitant.[3] There are several other facts, from the experiments with picryl chloride, pointing in this direction. In the first place it has not been possible to induce skin sensitivity to the simple substance, although an anaphylactic state is set up in this way, by injecting the protein conjugate intradermally, in contrast to the outcome of the converse experiment (Table I). Again, several attempts at passively sensitizing the skin to contact with the simple substance, by means of sera containing anaphylactic antibodies, have failed. Then in some experiments we observed a lack of parallelism in the degrees of anaphylactic and skin contact sensitizations in animals prepared by intradermal injections of picryl chloride. There are, finally, the desensitization experiments with protein conjugates (Table III) which were successful so far as anaphylaxis is concerned but were without effect on the dermal reactions produced by superficial application of the incitant to the intact skin. It would be premature to elaborate hypotheses concerning the differences in the processes leading to the two sorts of sensitization; tentatively it might be considered that the cutaneous manifestations are due to antibodies, perhaps of a special sort, produced by and fixed in the skin (*cf.* 6), or one could possibly think of the formation of various sorts of antigenic conjugates having the same "hapten component." The answer to these and other possibilities must await further study. Yet it would be most unlikely that the two specific sensitization effects induced at the same time by intradermal injections of "non-antigens," namely skin sensitivity and general anaphylaxis, are without a fundamental relationship.

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[3] *Cf.* Landsteiner and Levine (6), page 353; Landsteiner (4).

That the two conditions are related is strongly indicated by the fact that both were found to be induced by substances characterized by their ability to form conjugates (2). Antibodies, it is true, have so far been demonstrated in our experiments only for the anaphylactic sensitization which results from intracutaneous (or even superficial) treatment with suitable chemical substances. However, in the experimental allergic dermatitis of guinea pigs as in human contact dermatitis the instrumentality of antibodies in the broadest sense of the word, namely specific substances formed in consequence of previous contact,[4] must be assumed *a priori* because of the phenomenon of specificity, although they have not as yet been experimentally established. Considering other cases, such as the absence of circulating antibodies in later stages of the anaphylactic state, it is obvious that failure to demonstrate passive transfer of skin manifestations by means of serum is no decisive proof against the existence of antibodies, confined to the skin or perhaps circulating in small amounts and only transiently. Thus from the foregoing one may conclude that in the cases examined in this and in previous studies (1–3) the immunizing activity of conjugated antigens comes into play, this concept affording a plausible explanation for the immunological effects of simple substances.

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[4] *Cf.* Doerr (21, 22); Zinsser (23).

SUMMARY

It has been shown that by the cutaneous administration of simple chemical compounds in small quantities—2:4:6 trinitrochlorobenzene (picryl chloride) and 2:4 dinitrochlorobenzene, the latter a typical incitant of contact dermatitis in man—it is possible to induce true anaphylactic sensitization in guinea pigs, demonstrable by the intravenous injection of protein conjugates and by the Dale technique, using isolated uterine horns. This furnishes strong evidence for the formation of antigenic conjugates following application of substances of simple chemical constitution. Since the anaphylactic state is induced by the same method of administration that gives rise to cutaneous sensitivity, the assumption would appear justified, when one takes into account the chemical properties of the inciting substances, that the formation of conjugated antigens offers an explanation for the skin effects also.

In the experiments with picryl chloride, anaphylactic antibodies, and occasionally precipitins, have been demonstrated.

The differences between the cutaneous and anaphylactic types of sensitivity are discussed.

BIBLIOGRAPHY

1. Landsteiner, K., and Jacobs, J., *J. Exp. Med.*, 1935, **61**, 643.
2. Landsteiner, K., and Jacobs, J., *J. Exp. Med.*, 1936, **64**, 625.
3. Landsteiner, K., and Jacobs, J., *J. Exp. Med.*, 1936, **64**, 717.
4. Landsteiner, K., *New England J. Med.*, 1936, **215**, 1199.
5. Landsteiner, K., *J. Exp. Med.*, 1924, **39**, 631; and *Kong. Akad. Wetensch.*, Amsterdam, 1922, **31**, 54.
6. Landsteiner, K., and Levine, P., *J. Exp. Med.*, 1930, **52**, 347 (bibliography).

7. Coca, A. F., Walzer, M., and Thommen, A. A., Asthma and hay fever in theory and practice, Springfield, Ill., Charles C. Thomas, 1931, 62.
8. Klopstock, A., and Selter, G. E., *Klin. Woch.*, 1927, **6**, 1662; *Z. Immunitätsforsch.*, 1929, **63**, 463.
9. Fierz, H. E., Jadassohn, W., and Stoll, W., *J. Exp. Med.*, 1937, **65**, 339.
10. Wedrow, N., *Arch. Dermat. u. Syph.*, 1928, **154**, 143.
11. Wedroff, N. S., *Arch. Gewerbepath. u. Gewerbehyg.*, 1932, **3**, 509.
12. Wedroff, N. S., and Dolgoff, A. P., *Arch. Dermat. u. Syph.*, 1935, **171**, 647.
13. Dale, H. H., in A system of bacteriology in relation to medicine, London, His Majesty's Stationery Office, 1931, **9**, 229.
14. Coca, A. F., *Z. Immunitätsforsch.*, 1914, **20**, 622.
15. Doerr, R., and Bleyer, L., *Z. Hyg. u. Infektionskrankh.*, 1926, **106**, 388.
16. Holleman, A. F., *Rec. trav. chim. Pays-bas*, 1904, **23**, 253.
17. Bristol, P., and Fleischner, E. C., *Proc. Soc. Exp. Biol. and Med.*, 1925, **22**, 258.
18. Fleisch, A., *Arch. exp. Path. u. Pharmakol.*, 1922, **94**, 22.
19. Kobayashi, Y., *Japan. J. Dermat. and Urol.*, 1935, **37**, 92. [Abstract.]
20. Blank, J. M., and Coca, A. F., *J. Allergy*, 1936, **7**, 552.
21. Doerr, R., *Schweiz. med. Woch.*, 1921, **51**, 939.
22. Doerr, R., in Kolle, W., and von Wassermann, A., Handbuch der pathogenen Mikroorganismen, Jena, Gustav Fischer, 3rd edition, (Kolle, W., Kraus, R., and Uhlenhuth, P.), 1929, **1**, Liefg. 29, 936.
23. Zinsser, H., Resistance to infectious diseases, New York, MacMillan, 4th edition, 1931, 450.

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SPREADING PROPERTIES OF LEECH EXTRACTS AND THE FORMATION OF LYMPH

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Plate 9

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Extracts of certain mammalian organs, notably the testicle, have the power to increase tissue permeability (1). The rapid fading out of the wheal of injection when such an extract has been injected contrasts with the persistence of the bleb which follows the introduction of a fluid not possessed of the spreading property. The way in which tissue permeability comes to be modified is not yet understood. The permeated skin shows minor histological changes, which are restricted to the corium, the epithelial components remaining unaffected. Injection of highly concentrated testicle extracts produces, in addition to the spread, a local edema (2). This fact suggests that the phenomenon of spread may be related in some way to the processes which control the formation and removal of lymph.

Heidenhain endeavored to prove that lymph was a product of secretion of the endothelium, and not the result of mere filtration through the vessel walls. He found that certain substances, for example, peptone, egg albumin, extracts of liver and intestine, but more especially, extracts of mussels, leeches, and crabs, would produce a lasting increase in the lymph flow though they had no effect on the blood pressure. He termed such substances lymphagogues of the first class (3). A second group of lymphagogues included substances which increased the lymph flow from the thoracic duct by raising the pressure of the blood. In view of the possible relationship between the spreading phenomenon and the formation of lymph, some of Heidenhain's lymphagogues have been tested. In a preliminary investigation it was noted that egg albumin failed to produce a spread on injection into the skin and that one make of commercial peptone had a slight effect, whereas another was completely inactive. A number of lymphagogues of the second class, including concentrated solutions of sodium chloride and sugar, caused no appreciable spread. Leech extracts, on the other hand, had a marked effect and the analysis of this fact has provided the material for the present communication.

Material and Methods

Preparation of Leech Extract.—The medicinal leech was used as the source of the extract. In some tests the crop was partially emptied of the blood previously ingested by leaving the leech in contact with crystals of sodium chloride or with a concentrated salt solution. The bodies were washed thoroughly, first with tap water and then with distilled water. They were then cut into small pieces and passed once or twice through a Latapie masher. The resulting pulp was ground with sand and extracted with a volume of distilled water equal to six times the weight of the pulp. After centrifugation, the supernatant fluid was filtered through coarse paper. The total solid content of such extracts was about 12 to 18 mg. per cc.

The testicle extract used in some tests for comparison was prepared in the manner already described (2). The extraction was made with a volume of distilled water representing six times the weight of the pulp.

Determination of Spreading Power.—The power of an extract to spread was determined by measuring the area of diffusion 24 hours after the rabbit had been injected intradermally with the test solution mixed with India ink. The ink used as indicator was Higgins' India ink diluted 1:3 with water and filtered through a Berkefeld candle. The area of spread depends to a large extent on the individual permeability of the rabbit skin, which varies greatly from one animal to another. In an attempt to take these variations into account, the area of spread produced by the extract was divided by the area of spread produced by a control, in this case saline or Ringer's solution, giving an index of diffusion more or less independent of the individual permeability of the skin.

The relation which exists between the extent of the spread and the concentration of the factor in solution is not of a simple order. For instance, the area of spread normally produced by the undiluted extract may be reduced no more than one-half when a tenfold dilution of the extract is injected. This effect may be misleading when one has to compare the relative potency of different preparations obtained from the fractionation of an active extract. The difficulty may be partially obviated by testing repeatedly the solution at various dilutions and comparing the solutions giving equal spread. Such solutions may be assumed to contain an equal quantity of spreading factor.

Spreading Properties of Leech Extract.—Preliminary tests had indicated that leech extract increased the permeability of the skin in a manner similar to that observed for testicle extract, but more markedly. The following experiments illustrate the comparative spreading power of the two agents.

For the tests 0.5 cc. of leech extract, prepared in the manner described above, and 0.25 cc. India ink indicator were mixed in the syringe and injected intracutaneously into the upper part of the flank of a rabbit. The first indication of the action of the solution was the lack of resistance as the injection proceeded, and the immediate flattening of the bleb thereafter. Dispersion of the ink particles through the dermis, a process which continued for several hours, was relatively rapid, the main direction of the spread being determined by gravity. 20 hours after the injection, the area of spread, calculated from four similar experiments, averaged 120.6 sq. cm. The spreading mixture was found to have reached the lower part of the abdomen, extending to the mid-ventral line, and as far back as the scrotum. The skin of the involved area was thickened and edematous.

TABLE I

Effect of Leech and Testicle Extracts on the Permeability of the Rabbit Skin

Extract tested	Area of spread of 0.5 cc. extract plus 0.25 cc. India ink indicator	Area of spread of 0.5 cc. saline plus 0.25 cc. India ink indicator (control)	Ratio of active spread to spread of control
	sq. cm.	*sq. cm.*	
Leech	120.6	5.0	24.1
Leech (1:50 dilution with saline)	15.7	4.1	3.8
Rat testicle	14.3	5.0	2.9
Bull testicle	18.3	5.0	3.7

Autopsy showed large amounts of a somewhat gelatinous fluid accumulated in the subcutaneous tissue of the lower part of the abdomen and of the testicle.

The results of four experiments are summarized in Table I. For comparison, the effects of the diluted leech factor, and of rat and bull testicle extracts, prepared in the same way and with the same proportion of fluid to tissue, are included in Table I. Fig. 1 illustrates the relative spreading power of leech and of bull testicle extracts. The accumulation of fluid in the subcutaneous tissue, as a result of the injection of the leech extracts, would suggest that the leech factor, in at least one phase of its activity, affects the exchange of fluids in the living tissues.

The fact that the leech extract, diluted 50 times, is practically as active as the undiluted testicular extract would indicate that the spreading factor is 50 times more concentrated in the standard leech extract, or that the leech factor is a chemically different substance, endowed with a spreading power considerably greater than that of the factor from testicle. With this problem in view, the chemical properties of the leech factor have been investigated.

Chemical Properties of the Leech Spreading Factor

Experiments on Solubility.—The leech spreading factor is readily soluble in water. Aqueous extracts of different parts of the leech (head alone, or body separated from the head) were brought to pH 4.5 by the cautious addition of 0.1 N acetic acid. An abundant precipitate separated, and another precipitate was formed when the acid solution was neutralized by means of N NaOH. The results of the tests in the rabbit skin are given in Table II. It shows that, after removal of the two precipitates, no more than 10 to 20 per cent of the factor originally present was retained in the solution.

In the next experiment, an attempt was made to extract the factor directly with weak acid solutions. The pulp prepared by grinding the whole leech body was extracted with a volume of 0.1 N acetic acid equivalent to six times the weight of the tissue. A second lot was treated in the same way with 0.05 N acetic acid. The reaction of the two extracts after centrifugation and filtration was pH 4.0 and pH 4.4, respectively. The use of acid solution instead of water resulted in a marked reduction in the solid content of the extracts, which was 9.9 mg. per cc. for the 0.05 N acetic acid extract, against 18.2 mg. per cc. for the water extract. The spreading power of these preparations is given in Table II. It shows that the spreading factor is soluble in weak acid, but the water extract was regularly more active. However, the loss in spreading power seemed to be compensated by a parallel loss in inert matter. When the spreading power was compared in terms of dry weight, the acid extract appeared to be at least as active as the neutral extract, giving a spread of 9.6 sq. cm. per mg. of solids as compared to 7.6 sq. cm. for the water control.

The factor is not soluble in acetone. An aqueous extract was treated with acetone, and the precipitate collected and dried in air. The acetone filtrate was evaporated to dryness. Extracts prepared from the two fractions were tested for spreading power. The acetone soluble fraction was inactive, and no more than 40 per cent of the original activity was recovered from the precipitate. When the results were calculated on the basis of dry weights, the extract from acetone precipitation was found to represent a purer product in that the spread obtained was 8.5 sq. cm. per mg. of solids, against 2.5 sq. cm. for the watery extract. The absolute loss in spreading power suggests that the factor is partially denatured by acetone.

Experiments on Filtration.—The leech factor readily passes a Berkefeld filter, but is completely held by a collodion membrane which retains proteins. The fresh extract of the leech proved to have little diffusible matter, as shown by the fact that the dry weight was reduced only from 18.3 to 15.8 mg. per cc. after 5 hours dialysis in cellophane sacs, impermeable to proteins, on the shaking apparatus of Northrop and Kunitz (4). This treatment caused no reduction in the activity of the solution.

TABLE II

Solubility in Weak Acid of the Spreading Factor of Leech Extracts

Material extracted	Solvent	pH of extract	Treatment of extract	Spread in the rabbit skin		
				Area of spread of 0.5 cc. extract plus 0.25 cc. India ink indicator	Area of spread of 0.5 cc. saline plus 0.25 cc. India ink indicator	Ratio of active spread to spread of control
				sq. cm.	*sq. cm.*	
Leech head	Distilled water	6.9	—	96.5	5.3	18.2
	" "	6.9	Brought to pH 4.5 then neutralized	51.0	5.3	9.6
Leech body (head excluded)	Distilled water	7.1	—	37.4	3.4	11.0
	" "	7.1	Brought to pH 4.5 then neutralized	22.1	3.4	6.5
Entire leech	Distilled water	7.0	—	65.8	1.5	43.8
	N:10 acetic acid	4.0	Made neutral	40.7	1.5	27.1
Entire leech	Distilled water	7.0	—	139.7	5.5	25.4
	N:20 acetic acid	4.4	Made neutral	95.0	5.5	17.3

Inactivation by Heat.—A leech extract, prepared in the usual way, and diluted 1:2 with Ringer's solution, was heated by immersion in boiling water for 6 minutes. The resulting precipitate was removed and the clear supernatant fluid tested at various dilutions in the rabbit skin. The results are shown in Table III.

The fact that the area of diffusion of the heated extract at the lowest dilution was not greater than that of the unheated extract, 50 times more diluted, indicates that no more than 2 per cent of the original spreading power was left after the heating.[1]

The fact that 90 to 98 per cent of the spreading power is lost upon heating a crude extract at 95°C. for 5 to 15 minutes, was demonstrated repeatedly in additional experiments. The spread produced by the heated extracts was usually delayed and progressed slowly, as in the case of material of low grade activity.

TABLE III

Effect of Heat on the Spreading Power of Leech Extracts

Test No.	Solutions tested	Dilutions tested	Solids in solution	Area of spread of 0.5 cc. saline plus 0.25 cc. India ink indicator	Non-heated extract		Extract heated at 95°C.		Physical changes on heating
					Area of spread of 0.5 cc. solution plus 0.25 cc. India ink indicator	Ratio of active spread to spread of control	Area of spread of 0.5 cc. solution plus 0.25 cc. India ink indicator	Ratio of active spread to spread of control	
			mg. per cc.	*sq. cm.*	*sq. cm.*		*sq. cm.*		
1	Leech extract (1:6)	1:2	5.90	6.7	66.7	10.0	40.7	6.0	Precipitate formed
	" "	1:20	0.60	6.7	58.8	9.0	23.5	3.8	" "
	" "	1:100	0.12	6.7	39.2	5.8	14.8	2.2	" "
2	Leech heads extract (1:20)		5.0	5.9	41.0	7.0	31.2	5.3	Remains clear

Extracts prepared from the leech head are usually purer than those prepared from the whole body. In the next experiment, leech heads were extracted with a volume of water equivalent to 20 times the weight of the tissue pulp. This extract, after centrifugation and filtration through paper, was immersed in boiling water for 15 minutes. In this case no precipitate developed to complicate the interpretation of the test. The results recorded in Table III show that, even in the absence of flocculation, the spreading power of the solution was considerably reduced by heat, indicating a direct action rather than a secondary inactivation by adsorption on a precipitate.

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[1] Incidentally, these results illustrate the point, discussed above, that the area of spread is not directly proportional to the concentration of the active factor.

Separation by Copper Sulfate.—In the repeated attempts to find a simple method for the purification of the leech factor, it was found that copper sulfate would precipitate large amounts of inert material without reducing the spreading power of the extract. A 0.5 per cent copper sulfate solution was added drop by drop to a water extract of leech heads until the reagent no longer produced further precipitation. After removal of the precipitate by filtration on paper, the clear solution was dialyzed against cold, distilled water on the shaking machine for 8 hours. Test showed that precipitation and dialysis had removed as much as 89.2 per cent of the solids of the original extract, the precipitation with copper having accounted for 60 per cent of the reduction. The final product, tested in the rabbit skin, showed a spreading power greater than that of the original extract. The relative spreading power, expressed in terms of dry matter in solution, was 6.3 sq. cm. per mg. solids for the untreated extract against 59.0 sq. cm. for the purified fraction. The results are shown in Table IV.

TABLE IV

Effect of Copper Sulfate on the Spreading Power of Leech Extracts

Solutions tested	Characters of extracts	Total solids	Spread in the rabbit skin		
			Area of spread of 0.5 cc. solution plus 0.25 cc. India ink indicator	Ratio of active spread to spread of control	Area of spread per mg. solids in solution
		mg. per cc.	*sq. cm.*		*sq. cm.*
Leech heads extract...........	Dark brown, turbid	14.7	93.0	17.0	6.3
Copper sulfate filtrate.........	Clear, colorless	5.5	110.4	20.0	20.0
Copper sulfate filtrate, dialyzed.	" "	1.6	94.6	17.1	59.0
Copper sulfate, 0.5 per cent solution (control)...........	—	—	5.5	—	—

The increase in spreading power, after treatment with copper sulfate, is difficult to explain unless we assume that the action of the reagent dissociated inhibiting elements from the spreading factor, or that copper sulfate reacted with it to form a more active compound. A 0.5 per cent solution of copper sulfate had no apparent effect on skin permeability.

In the foregoing tests the leech factor was found to be soluble in water and in weak acid, but was precipitated by acetone. It did not pass collodion or cellophane filters, which retain proteins. There is evidence that the factor is inactivated by heat. All active fractions from the leech gave a positive diazo reaction, similar in this respect to the active fractions separated from testicle (5). In these general properties the leech factor resembles the testicular factor which, in a recent study, was shown to present the characters of proteins. Elucidation of the chemical relationship which may exist between the spreading factor from the leech and that found in mammalian organs must await further experiments.

Relation between the Leech Anticoagulating and Spreading Factors

The anticoagulating properties of leech extracts have long been recognized (6). The occurrence of a spreading agent in the same extracts brought up the question of the possible identity of the two principles. The following experiments represent an attempt to dissociate the spreading from the anticoagulating factor.

As the anticoagulating factor is assumed to originate in the buccal cavity and the pharynx (7), extracts of the different parts of the leech body were prepared and tested for the two factors. The head, taking in the first 10 segments, was the source of one extract and the rest of the body of the other. In some tests the body was prepared before extraction by opening the crop and washing out the contents. Tests were also made of extracts prepared from the latter and from the gonads. The ovaries, spermatophores, and the nine pairs of testicles were extracted together. Before testing, the crop content was diluted up to six times its volume with water. In all cases the tissue extracts were prepared as described above with a volume of water representing six times the weight of the tissue. The results of the tests in the skin of rabbits are shown in Table V.

It will be seen from Table V that the greatest spread was obtained from the isolated head and gonads, these two fractions being about equal in effect. The spreading power exhibited by the crude body extract was practically lost when, prior to mincing and extraction, the organs were removed and the internal wall was washed with a stream of water. Washing the surface of the sex organs before extraction removed also much of the spreading factor. It can be inferred from this that the muscular sheet of the leech body contains no appreciable amount of the spreading factor, and that even an important part of that present in the extract of the unwashed gonads may come from another source. It seems unlikely that surface washing would remove the factor if it existed in the tissue. Body cavities and the sex organs located within the adjoining segments may conceivably be contaminated by the content of the pharynx when the head is removed. According to these results, the anterior digestive tract would appear to be the main source of the spreading factor as well as that of the anticoagulating factor. Other methods have been sought to separate the two.

TABLE V

Effects of Extracts from Different Parts of the Leech on Skin Permeability

Tissues extracted	Characters of the extracts	pH	Total solids	Area of spread of 0.5. cc. extract plus 0.25 cc. India ink indicator	Area of spread of 0.5 cc. saline plus 0.25 cc. India ink indicator (control)	Ratio of active spread to spread of control*
			mg. per cc.	*sq. cm.*		
Leech head..............	Light brown or greenish in color; clear or opalescent	6.9–7.1	13.1	91.2	5.0	18.2
Leech gonads............	Light brown; opalescent	6.9–7.1	—	80.7	4.9	16.4
Leech gonads (washed)....	Light brown; opalescent	6.9–7.1	7.9	41.6	7.4	5.6
Leech body..............	Dark brown to red in color; turbid	7.1	14.7	66.9	4.9	13.6
Leech body (washed).....	Dark brown to red in color; opalescent	7.1	8.0	21.0	7.4	2.8
Crop content (mainly ingested blood)..........	Dark red; clear	—	—	20.9	4.0	5.2

Spreading and Anticoagulating Power of Leech Extracts.—In the next series of experiments the leech extracts were tested for both spreading and anticoagulating properties. The anticoagulating power was determined by adding 0.5 cc. of leech extract to 2 cc. of fresh rabbit blood and recording the time of clotting.

As shown in Table VI, the extracts most active in retarding blood coagulation were also those endowed with the greatest spreading power. This parallelism was maintained when the leech extracts were heated by immersion in boiling water for 6 minutes. Purification by precipitation with copper sulfate and dialysis enhanced the spreading and anticoagulating properties of the extract. These results would seem to favor the view that both effects are produced by a single factor. This opinion is not supported by the results given in Table VII.

TABLE VI

Spreading and Anticoagulating Power of Leech Extracts

Test No.	Solutions tested	Dilution of original extract with saline	Area of spread of 0.5 cc. solution plus 0.25 cc. India ink indicator	Clotting time of 2 cc. fresh rabbit blood plus 0.5 cc. test solution
			sq. cm.	*min.*
1	*Extracts from various sources*			
	Leech head	1:20	61.6	100
	Leech body	1:20	44.0	14
	Leech gonads	1:20	32.3	9
	Saline (control)	—	6.6	5
2	Leech head	1:50	33.0	20
	Leech gonads (washed)	1:50	11.4	2
	Leech body (washed)	1:50	10.6	1
	Saline (control)	—	5.5	1
3	*Effect of heat on activity*			
	Leech head extract	1:600	39.2	33
	Leech head extract, heated	1:600	14.8	16
	Ringer's (control)	—	6.7	4
4	*Effect of copper purification*			
	Leech body extract	—	31.6	24
	Leech body extract after copper precipitation, and dialysis	—	37.0	Fluid after 12 hrs.
	Saline (control)	—		

In this experiment, commercial hirudin (6, 7) and fresh leech extracts were compared, the stock solutions being adjusted to contain 5 mg. matter per cc. The anticoagulating power of the hirudin preparation appeared to be even superior to that of the fresh extract of the same concentration, whereas its spreading power was low and was practically abolished by 1:10 dilution. This indicates that the spreading and anticoagulating properties may exist independently in the leech extracts.

From the foregoing experiments it appears that the spreading and anticoagulating factors have practically the same distribution in the body of the leech, originating for the most part in the anterior digestive tract.[2] It is still uncertain whether a single substance is endowed with both anticoagulating and spreading properties, although the existence of two factors appears to be probable. If two different factors are involved, they may be closely related chemically, a fact which would explain the difficulties encountered in their separation.

TABLE VII

Spreading and Anticoagulating Properties of Leech Extracts and of Commercial Hirudin

Solutions tested	Total solids in solution	Area of spread of 0.5 cc. solution plus 0.25 cc. India ink	Clotting time of 2 cc. fresh rabbit blood plus 0.5 cc. test solution				
			10 min.	15 min.	2 hrs.	10 hrs.	20 hrs.
	mg. per cc.	*sq. cm.*					
Hirudin	5.0	19.5	–	–	–	–	+ Clot not contracted
Leech head extract	5.0	54.5	–	–	–	–	+
Leech body extract	5.0	47.0	–	–	+	Clot contracted	
Hirudin (1:10)	0.5	6.5	–	–	+		
Leech head extract (1:10)	0.5	27.1	–	+			
Leech body extract (1:10)	0.5	16.3	–	+			
Saline (control)	–	5.8	+				

DISCUSSION

The equilibrium between tissue fluid and the blood is maintained by the capillary wall, acting as a semipermeable membrane, and the interplay of physical forces which give rise to filtration, osmosis, and diffusion. The theory which would refer the formation of lymph to these factors, first formulated by Ludwig and Starling, has received the support of modern investigators, and the observations that have accumulated on the subject are best interpreted in terms of this hypothesis (8–10). Heidenhain made the observation that certain substances, when introduced into the circulation, had the power to increase the lymph flow from the thoracic duct without exerting any definite action on the blood pressure (3). At the same time he noted that the content of organic matter in the lymph was augmented. He held these observations to demonstrate a secretory function of the capillary wall. The interpretation of Heidenhain is not accepted now and the facts are taken to indicate that the permeability of the endothelium of the vessels has been increased. The intimate changes induced in the capillary wall by Heidenhain's lymphagogues of the first group, especially by extracts of leech and mussels, seem not to have been investigated. The effect of leech extract on lymph formation brings up the question whether the phenomenon of spread may be caused, at least in part, by a sudden increase in vascular permeability. An excess of fluid filtering through the capillary wall and flooding the tissue spaces may separate widely the components of the connective tissue where solutes or particles, if present, would be dispersed passively. Against the view that a local increase in the permeability of the vessels is the only factor conditioning the spread, is the observation that testicular (11) and leech extracts will spread, although to a lesser extent, in a fragment of skin separated from the body.

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[2] It is conceivable that the spreading factor is released together with the anticoagulating factor at the moment of the bite. Professor L. Delrez has brought to our attention the fact that the therapeutic application of leeches is sometimes attended by extensive suffusion of blood in the subcutaneous tissue.

Azoproteins have been shown to spread when introduced into the dermis (12). Although the area of spread produced by the injection of leech extracts or azoproteins may be ultimately the same, there is evidence that the phenomenon is induced by a different mechanism in the two instances. During the active spread the skin injected with leech extract remains smooth and relatively flaccid, whereas that receiving azoproteins becomes thickened and appears to be under tension. The osmotic pressure of the azoproteins tested for spreading power was found to be considerably greater than that of the uncoupled proteins, which under the conditions of the experiments was negligible (12). The presence in the subcutaneous tissue of azoprotein solutions of high osmotic pressure may cause large quantities of fluid to filter from the vessels into the tissue spaces, until an equilibrium is reached, without affecting necessarily the normal permeability of the blood capillary. As a result the tissue spaces would become distended temporarily by the excess of fluid, allowing a passive dispersion of the substances which may have been introduced along with the azoproteins. Further experiments may be necessary to test this possible mode of action of azoproteins.

SUMMARY

1. The injection of leech extracts into the skin increases its permeability, as shown both by the spread of fluid and of foreign particles through the dermis. The spread is followed some hours after the injection by more or less edema of the subcutaneous tissue.

2. A preliminary study of the chemical properties of the leech spreading factor indicates a similarity with the spreading factor prepared from testicle.

3. Attempts to separate the leech spreading and anticoagulating factors showed that the two have practically the same distribution in the leech body, extracts from the separated head being the most active.

4. It is undetermined whether two distinct factors are responsible for the spreading and anticoagulating properties of leech extracts. A chemical similarity is suggested by the fact that agents which affect the activity of one factor have a parallel effect on the other.

5. The mechanism of the spread produced by leech extracts and by other spreading agents is discussed.

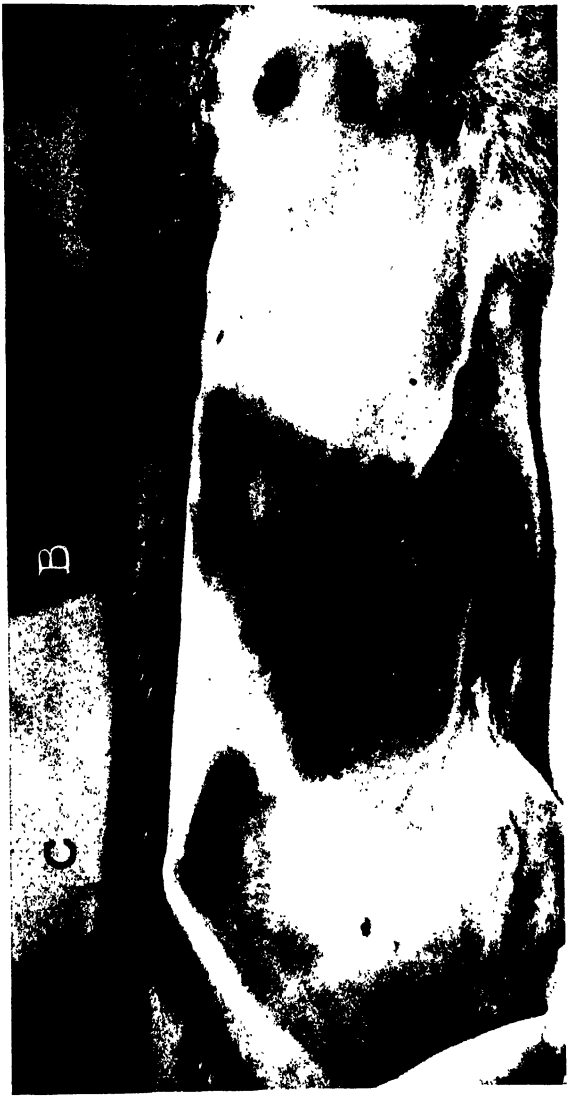
BIBLIOGRAPHY

1. Duran-Reynals, F., *Compt. rend. Soc. biol.*, 1928, **99**, 6; *J. Exp. Med.*, 1929, **50**, 327. McClean, D., *J. Path. and Bact.*, 1930, **33**, 1045. Hoffman, D. C., and Duran-Reynals, F., *Science*, 1930, **72**, 508; *J. Exp. Med.*, 1931, **53**, 387.
2. Claude, A., and Duran-Reynals, F., *J. Exp. Med.*, 1937, **65**, 661.
3. Heidenhain, R., *Arch. ges. Physiol.*, 1891, **49**, 209.
4. Northrop, J. H., and Kunitz, M., *J. Gen. Physiol.*, 1925–26, **9**, 351. Kunitz, M., and Simms, H. S., *J. Gen. Physiol.*, 1927–28, **11**, 641.
5. Claude, A., *Science*, 1933, **78**, 151.
6. Haycraft, J. B., *Proc. Roy. Soc. London*, 1883–84, **36**, 478.

7. Franz, F., *Arch. exp. Path. u. Pharmakol.*, 1902-03, **49**, 342. Parker, I. J., and Haswell, W. A., A textbook of zoology, London, MacMillan and Co., 3rd edition, 1921, **1**.
8. Starling, E. H., *J. Physiol.*, 1894, **16**, 224; 1895-96, **19**, 312; Principles of human physiology, Philadelphia, Lea and Febiger, 3rd edition, 1920.
9. Lazarus-Barlow, W. S., *J. Physiol.*, 1895-96, **19**, 418. Asher, L., and Barbera, A. G., *Z. Biol.*, 1898, **36**, 154.
10. Krogh, A., The anatomy and physiology of the capillaries, New Haven, Yale University Press, revised edition, 1929. Landis, E. M., *Physiol. Rev.*, 1934. **14**, 404.
11. McClean, D., *J. Path. and Bact.*, 1931, **34**, 459.
12. Claude, A., *J. Exp. Med.*, 1935, **62**, 229.

EXPLANATION OF PLATE 9

Fig. 1. Rabbit 9-99 (right side). The spread of India ink as influenced by: A, saline control; B, leech extract; C, bull testicle extract. The final measurements of the areas of spread were 6.6, 112.2, and 17.0 sq. cm., respectively. Under the action of the leech extract the ink particles spread extensively through the cutaneous tissue. Less than 24 hours after the injection, the mixtures of leech extract inoculated respectively on the two sides of the back had merged under the abdomen at the mid-ventral line. Ink particles were found accumulated along the abdomen as far back as the connective tissue of the scrotum. The subcutaneous tissue of the skin over the ventral surface of the abdomen was edematous.



Photographed by Joseph B. Haulenbeek

(Claude: Spreading properties of leech extracts)

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LIVER DEGENERATION AND CIRRHOSIS PRODUCED BY 1:2:5:6 DIBENZANTHRACENE

BY ALBERT CLAUDE, M.D.

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Coal tars and shale oils have been used extensively to induce cancer in mice, rats, rabbits, and fowl. The mortality among the animals treated is high, and an intense tissue reaction is usually produced at the site of contact. Davidson (1) showed that external application of tar to the ears of rabbits, or injection of an ether solution would produce a degeneration of the liver cells and cirrhosis. Polson (2) obtained the same effect by the use of shale oil. Since the materials employed were complex mixtures, it remained undetermined whether the carcinogenic power and the toxicity on the liver were associated properties of the same chemical. In the present study rabbits were treated with 1:2:5:6 dibenzanthracene, a substance which has been repeatedly demonstrated as a powerful carcinogenic agent. The subcutaneous injection of the chemical was followed by extensive damage to the liver parenchyma in many of the animals treated. This note is a report of the results obtained with the crude and the purified hydrocarbon.

In the first series of experiments, rabbits of various breeds were injected subcutaneously with a 0.4 per cent solution of commercial 1:2:5:6-dibenzanthracene in lard.[1] The injections, each of 10 to 15 mg. of the chemical, were made at weekly intervals for a first group of 3 rabbits, at monthly intervals for the other groups, with occasionally two to three month intervals for the rabbits surviving more than six months. Twelve out of a total of 18 rabbits died with signs either of acute liver necrosis or of chronic liver degeneration and cirrhosis. Of this group, 83.3 per cent suffered from ascites and edema in varying degree at the time of death. Individual resistance varied greatly, 6 rabbits living more than two and a half years, during which time they received large amounts of the hydrocarbon without any apparent ill effect, as shown by a steady increase in body weight. During this period one of these rabbits received 275.7 mg. dibenzanthracene in 16 injections, or approximately 1.0 mg. per 15 gm. body weight. At autopsy, the liver showed fatty degeneration, but no fibrosis.

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[1] The hydrocarbon was obtained from the Eastman Kodak Company.

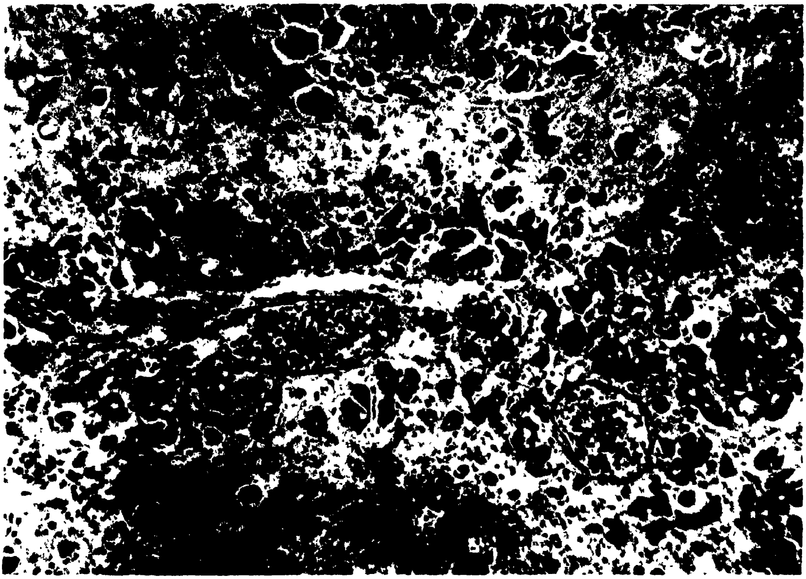

FIG. 1. Liver of rabbit 9–96 (hair brown) showing a portal tract surrounded by several layers of healthy liver cells.

On either side the centers of the lobules are completely necrotic. The animal received 5 mg. dibenzanthracene per 100 gm. body weight, over a period of seventeen months. Death occurred seventeen days after the last injection, with hobnail liver, edema, and ascites. Methylene blue-eosin. × 72.

Six control rabbits, injected with lard alone instead of the dibenzanthracene solution, were kept for eighteen months under the same conditions as the experimental animals. During this period, the average body weight increased from 2.8 to 3.9 kg. When killed, these animals were in excellent health and no lesions were found at autopsy.

Commercial dibenzanthracene is contaminated by a colored impurity which is not removed by recrystallization. In order to determine whether the effect on the liver was caused by the hydrocarbon itself, crude dibenzanthracene was purified by repeated washing of a toluene solution with concentrated sulphuric acid and further recrystallization of the substance until a colorless product was obtained. In the next experiment 4 rabbits were treated with a colloidal solution of this purified dibenzanthracene (3). A total of 11 injections were made at weekly intervals, each injection being adjusted to represent 5 mg. hydrocarbon per kilo of body weight. Three rabbits died six, twenty-one, and twenty-nine days following the last injection. At autopsy important degenerative changes of the liver were found, along

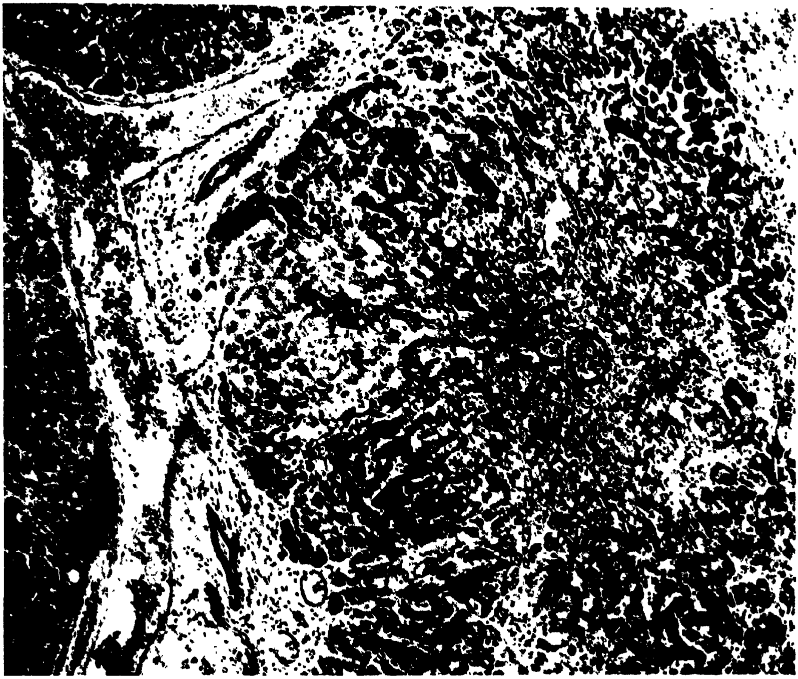

FIG. 2. Liver lobule of rabbit 9–35 (silver martin) with the central vein and secondary branches highly distended.

Vasodilatation parallels necrosis of liver cells, most of which have been removed. The portal vein and bile ducts are seen at the left. This animal received about 2 mg. dibenzanthracene per 100 gm. body weight, over a period of three and a half months. It died twelve days after the last injection, with edema and ascites. Methylene blue-eosin. × 80.

with edema of the skin and transudates in the peritoneal, pleural and pericardial cavities. The fourth rabbit was killed to end the experiment. There was no ascites, but there was a marked necrosis of the liver cells and congestion of the organ. About one-fifth of the total amount of dibenzanthracene injected was recovered by benzene extraction of the dried skin of the rabbits treated.[2] The same method failed to demonstrate the presence of dibenzanthracene in the benzene extracts of the liver, the bile or the ascitic fluid. The carcinogenic power of each batch of dibenzanthracene employed was tested in mice, 70 to 80 per cent of which died with sarcoma within a year.

FIG. 3. Rabbit 6, injected subcutaneously with a colloidal suspension of purified dibenzanthracene: Inoculum, six weeks after injection, showing congestion and petechiae in the underlying muscle sheet. Other lesions or reaction practically absent.

Microscopically the lesions produced in the rabbit liver by the crude or purified dibenzanthracene were those of acute necrosis when the injections were frequent, of cirrhosis with intermediate stages of degeneration and regeneration when time was allowed for the liver to recuperate between the injections. Massive destruction of liver cells occurred in the central zone of the lobules, with practically no transition between the necrotic area and the apparently normal cells surrounding the portal tracts (Fig. 1). In the necrotic areas, the liver cells appeared opaque and uniformly stained with eosin, the nuclei being absent or represented by pyknotic débris. Vasodilatation and congestion were frequent in the degenerated liver tissue, the necrotic areas being occupied by engorged blood vessels (Fig. 2). Passive distention of the central veins in the affected areas appeared to result from degeneration of the liver cells and subsequent removal of the necrotic material. When necrosis was more extensive and involved confluent lobules, hemorrhages occurred in the affected region and on the surface of the liver, which was covered with a thick layer of fibrin. When recovery occurred, there was produced the typical abnormal lobular architecture seen in toxic cirrhosis, with interstitial fibrosis and proliferation of the bile-ducts. These results suggest that the hydrocarbon, when introduced subcutaneously, may be eliminated through the liver, although no dibenzanthracene could be demonstrated in the liver or the bile extracts.[a] Other lesions presented by the rabbits treated were degeneration of the parenchyma of the lymph nodes and the spleen. The spleen showed intense engorgement of the pulp sinuses, hemorrhage into the malpighian follicles and punctate necrosis of lymphocytes. Hemorrhage may have been the cause of a marked pressure atrophy of malpighian bodies. Much pigment was often present, as seen in severe hemolytic anemia. Lymph nodes showed lesions similar to those encountered in the spleen. At the site of inoculation the most obvious changes were on the blood vessels, as shown by vasodilatation and petechiae (Fig. 3).

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[2] The spectrographic determinations were made by Dr. A. Rothen.

REFERENCES

1. Davidson, J.: J. Path. & Bact. 28: 621, 1925.
2. Polson, C. J.: J. Path. & Bact. 34: 5, 1931.
3. Boyland, E.: Lancet 2: 1108, 1932.
4. Chalmers, J. G., and Peacock, P. R.: Biochem. J. 30: 1242, 1936.

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[a] These results are in agreement with those of Chalmers and Peacock (4) who found that dibenzanthracene injected intravenously disappeared rapidly from the blood but its fate, beyond the liver, could not be ascertained.

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STUDIES ON THE RELATION BETWEEN TUMOR SUSCEPTIBILITY AND HEREDITY[1]

VI. LUNG TUMORS IN MICE WITH RESPECT TO THE PHENOMENON OF MATERNAL INFLUENCE

BY CLARA J. LYNCH, PH.D.

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The phenomenon known as maternal influence and its relation to the incidence of tumors in mice is being discussed widely at the present time. It was first reported as found in connection with the mammary tumors seen in these animals. Observations on this point were made independently in two laboratories: in 1933, in a preliminary note by the staff of the Roscoe B. Jackson Memorial Laboratory (1), attention was called to the difference in results given by reciprocal crosses between strains of mice; and about the same time, Korteweg, working with the same strains sent him in Amsterdam, had also observed this difference (2). Subsequently more extensive data have appeared from both these laboratories (3, 4). The phenomenon has been reported also in connection with leukemia and longevity in mice, and MacDowell and later MacDowell and Richter have submitted a large amount of data bearing on this subject (5).

In the phenomenon of maternal influence, as the name implies, a predominant part is played by the mother of the individuals under observation. In a cross, when the female parent comes from a susceptible strain, the incidence of disease among the offspring is greater than when she belongs to an insusceptible strain. Various suggestions have been made in explanation of this finding. The effect has been attributed to some property of the cytoplasm, or it has been associated with nursing, with the transfer of sex hormones, or with virus, or has been ascribed to certain other possibilities; but whatever may be the mechanism involved, it is obvious that the phenomenon has a fundamental bearing on the question of the hereditary transmission of susceptibility. Our laboratory has already submitted evidence to show that susceptibility to lung tumor in mice is affected by extrinsic factors but also, very clearly, by heredity. It is pertinent, therefore, to examine the data from the standpoint of maternal influence.

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[1] Read before the American Association for Cancer Research, Chicago, Ill., March 24, 1937.

The experiments to be presented in this paper involve crosses between three strains of mice, one with a low and two with higher incidences of lung tumor. The strains are: No. 1194, a colored (agouti) stock; an albino strain descended from mice obtained from Dr. H. J. Bagg; and another albino strain called D. The series of experiments stretched over a considerable period of time. A few date from 1925 but the majority were done between 1930 and 1933. Those involving Strain D were done in 1932–34.

The records show that the least susceptible strain of the three is No. 1194. The total incidence of lung tumor exhibited by this strain is about 2 to 4 per cent. Figures for Strain 1194 published in 1926 (6) showed that in 208 mice living a year or longer there was an incidence of only 6.7 per cent of tumor.

On the other hand, mice from the Bagg substrain, of comparable age, had a 37.0 per cent incidence of tumors in the lung. In this first survey no mice in either strain under twelve months of age had tumor. Later, however, tumors appeared in the Bagg strain in younger mice, and the records again showed a significant difference between these strains. Figures from the two surveys have been combined and the totals for mice over a year old are given in Table I.

The D strain is the most susceptible of the three under discussion. Although the tumor incidence was high at the outset, an attempt was made to increase it still further through selective inbreeding. Subsequently, tumors were noted in mice less than six months of age; and in the group of individuals which may serve for comparison with the experiments reported here, among 152 mice over twelve months, 66.4 per cent were tumor bearers.

These figures apply to the so-called "spontaneous tumors," but the incidence can be increased when the tumors are induced by cutaneous tarring. In this method of treatment, tar is applied to the skin at 12 different loci consecutively, three times a week for four months; and after a suitable interval, usually six months, when the animals are about a year old, they are killed. After this treatment the percentage of tumors usually increases, but not equally in all strains—differences are still apparent. Three hundred and twenty-nine mice from the least susceptible strain, after tarring, have given 20.1 per cent of lung tumor, whereas 443 Bagg albinos have given 84.4 per cent. Animals from Strain D have proved even more susceptible. One lot was killed two months after the cessation of tarring, when the mice were about nine months old; yet of the 90 individuals which survived the treatment only one failed to show a tumor (98.9 per cent susceptibility).

Since these strains provide suitable test material it is desirable to examine the reciprocal crosses made between the colored (agouti) strain and each of the two albino stocks (Bagg and D), with the idea that a comparison of the incidence of tumor produced in the resulting progeny may give information in regard to the possible operation of the factor of maternal influence. The observations have been made upon tumors induced by tar. Although the percentage of tumor is affected by tarring, since strain differences are maintained, tar-induced tumors may legitimately be used as a basis for comparison.

TABLE I

Percentage of Lung Tumor in Three Strains of Mice

Strain	Spontaneous			Tar-induced		
	Number of mice	Age	Per cent tumor	Number of mice	Age	Per cent tumor
1194	375	13–36 mos.	5.9	329	12–13 mos.	20.1
Bagg derivative	262	13–36 mos.	39.3	443	12–13 mos.	84.4
D	152	13–36 mos.	66.4	90	9 mos.	98.9

Results of the cross of Strain 1194 to the most susceptible line, Strain D, are shown in Tables II and III. The largest numbers of offspring resulted from the cross in which the female parent came from the high-tumor strain. The 31 females and 8 males of the parental generation were not tarred and attained various ages before the intervention of natural death. Three females were destroyed by cage mates. In a few cases it was not possible to distinguish histologically between primary and secondary growths in the lung, but about half the individuals of the original group were known to have primary lung tumors. Of the males belonging to the low-tumor strain, one was eaten by cage mates; the remaining 7 were tumor-free at autopsy. In the first generation of progeny resulting from these matings there

TABLE II

Crosses between Females from a Low-Tumor and Males from a High-Tumor Strain

Exp.	Number of Parents			Number of Offspring (tarred)			
	Females from Strain D		Males from Strain 1194	Males		Females	
				Tumor	No tumor	Tumor	No tumor
1142	4	×	1	28	2	17	0
1172	4	×	1	30	1	25	0
1191	5	×	1	18	1	26	0
1192	4	×	1	16	2	23	0
1220	3	×	1	11	1	6	0
1225	4	×	1	31	6	43	2
1230	4	×	1	21	1	31	1
1235	3	×	1	15	3	13	0
Totals	31		8	170	17	184	3
Total				354 tumor: 20 non-tumor (94.7 per cent tumor)			

TABLE III

Crosses between Females from a Low-Tumor and Males from a High-Tumor Strain

Exp.	Number of Parents			Number of Offspring (tarred)			
	Females from Strain 1194		Males from Strain D	Males		Females	
				Tumor	No tumor	Tumor	No tumor
1142D	1	×	1	9	0	3	0
1192D	1	×	“	6	0	0	0
1172D	1	×	1	5	0	3	0
1230D	1	×	“	10	0	6	0
1235D	1	×	1	3	0	4	0
Totals	5		3	33	0	16	0
Total				49 tumor: 0 non-tumor (100 per cent tumor)			

was a high incidence of lung tumors. The totals show that among 374 mice of both sexes, 94.7 per cent had tumor (Table II). It may be noted that the percentage was greater among the females than among the males, and to an extent that is mathematically significant. Our interpretation of this is uncertain, and in many cases we have found males more susceptible than females.

In the reciprocal cross the females came from the low strain, and on the theory that the mother has a predominant influence, the incidence of tumor among the offspring should be low. Data are available from only 5 females of this type. None of them had lung tumor, although the animals lived from twelve to seventeen months; but 2 of the 3 males selected as mates from the high-tumor Strain D had tumor. Of the 49 offspring (shown in Table III), none was free of tumor.

Although the number of animals in the second cross is small, it is sufficient to give an indication of the general relationships, and it is seen that the offspring of mothers from a low-tumor strain did not have a lower percentage of tumors than did the offspring from the reciprocal cross. In making these comparisons no corrections need be made for age variation, since the animals were all killed at one time. Mice dying without tumor before the end of the test period were omitted.

The remainder of the evidence to be presented involves the progeny from crosses between Strain 1194 and our Bagg substrain, and in addition the results from back-crosses between them and their first generation hybrids. In the first cross (Table IV) the female parents came from the high-tumor-bearing strain (Bagg). Of the first 8 females listed, only one (possibly 2) had lung tumor; the remainder did not show malignant growths in the lung, but the majority of them did not attain the average tumor age. The parents in the last experiment (H IV series) were tarred and all 6 females had tumor. All of the males in Table IV were from the less susceptible strain and were tumor-free except one, which had been tarred. In all cases the tarring was carried out after the young had been produced, hence the latter could not have been affected by the treatment. Again the progeny showed a high incidence of tumor—89.1 per cent among 92 mice. The percentage of tumors was somewhat higher in the females

(93.6 per cent) than in the males (84.4 per cent), but not significantly so.

TABLE IV

Crosses between Females from a High-Tumor and Males from a Low-Tumor Strain

Exp.	Number of Parents			Number of Offspring (tarred)			
	Females from Strain Bagg		Males from Strain 1194	Males		Females	
				Tumor	No tumor	Tumor	No tumor
1142B	3	×	1	4	2	6	0
1192B	3	×	1	15	1	20	2
1235B	2	×	1	10	0	13	0
HIV-54	1	×	1	2	0	0	0
HIV-56	1	×	1	1	2	4	0
HIV-55	1	×	"	4	2	0	0
HIV-59	1	×	1	1	0	1	0
HIV-60	1	×	"	1	0	0	0
HIV-53	1	×	1	0	0	0	1
Totals	14		7	38	7	44	3
Total				82 tumor: 10 non-tumor (89.1 per cent tumor)			

TABLE V

Crosses between Females from a Low-Tumor and Males from a High-Tumor Strain

Exp.	Number of Parents			Number of Offspring (tarred)			
	Females from Strain 1194		Males from Strain Bagg	Males		Females	
				Tumor	No tumor	Tumor	No tumor
A	5	×	1	20	2	19	0
B	4	×	1	25	0	17	2
HIV-1	1	×	1	1	0	1	0
HIV-16	1	×	1	4	0	0	0
HIV-29	1	×	1	1	1	0	0
HIV-9	1	×	1	1	0	0	0
Totals	13		6	52	3	37	2
Total				89 tumor: 5 non-tumor (94.7 per cent tumor)			

In the reciprocal cross all the parents were tarred (Table V). Of the 13 females from the low tumor strain, 8 died before the end of tarring, but 2 of those that lived through the treatment had tumor. Of the males, 3 had tumor; the others died early. Although the mothers in this cross came from the strain with low incidence, the progeny had as many tumors as did those from the opposite cross, in fact slightly more (94.7 per cent in 94 mice). Thus it was found that as far as lung tumors are concerned, reciprocal crosses between the Bagg substrain and Strain 1194 gave results similar to those obtained with Strains D and 1194, *i.e.*, these crosses failed to reveal any predominant influence exercised by the mother.

Attention may be called to the fact that in these two crosses between the Bagg and 1194 strains, several of the individuals from the low-tumor strain actually did have tumors. It would, perhaps, be proper to exclude them from the evidence. However, if the offspring from these mice are omitted from the total, the results of the comparison are not essentially changed (Table VI).

TABLE VI

Reciprocal Crosses between High-Tumor and Low-Tumor Strains, Omitting Cases in Which the Parent from the Low-Tumor Strain Had Tumor

Parental strain	Number of Offspring (tarred)						
Females Males	Males		Females		Total		Per cent tumor
	Tumor	No tumor	Tumor	No tumor	Tumor	No tumor	
Bagg × 1194	36	7	43	3	79	10	88.8
1194 × Bagg	47	3	29	2	76	5	93.8

Evidence in respect to the existence of maternal influence is furnished not only by parental crosses but also by back-cross matings. The experiments include three types of back-crosses, and it may be well to refer at this point to the formulae used by Little and his associates to represent these types (Table VII). C is the chromosomal or genic contribution, capital C if it is derived from a high-tumor strain, small c if from a low-tumor or non-tumor strain. The extrachromosomal influence is contributed by females only, and is represented by E if contributed by females from a high-tumor strain and by e if contributed by females from a low-tumor strain. In two of the back-crosses F_1 females are used. They are identical in constitution, and since the original female parent which they came from was from the low-tumor strain, they or at least a large majority of them would not have E (the extrachromosomal influence making for tumor susceptibility). Mated with males from either a high-tumor or a low-tumor strain, they would be expected, on the extrachromosomal hypothesis, to give progeny with a low incidence of tumor. On the genic hypothesis, F_1 hybrid females, whatever their origin, should give offspring with a higher incidence of tumor when mated with males from a high-

TABLE VII

Formulae Used by Murray and Little in Connection with the Phenomenon of Maternal Influence; and Certain Theoretical Expectations

Type of backcross (Original ♀ parent from low tumor strain)	Expected tumor percentage	
	Non-genic hypothesis	Genic hypothesis
F_1 ♀ $\frac{ce}{C}$ × ♂ $\frac{C}{C}$ (high tumor strain)	Low	High
F_1 ♀ $\frac{ce}{C}$ × ♂ $\frac{c}{c}$ (low tumor strain)	Low	Low
F_1 ♂ $\frac{c}{C}$ × ♀ $\frac{CE}{C}$ (high tumor strain)	High	High

C = chromosomal material derived from high-tumor strain. c = chromosomal material derived from low-tumor strain. E = extrachromosomal influence derived from high-tumor strain. e = extrachromosomal influence derived from low-tumor strain. The contribution of the female is shown as the numerator of a fraction. The contribution of the male is shown as the denominator of a fraction.

TABLE VIII

Back-Crosses of F_1 Individuals to Mice from Parental Strains

(Data from Exps. A and B and one mating from HIV)

Type of Back-cross (Original parent from Strain 1194)	Number of Parents		Number of Offspring (tarred)			Expected tumor percentage	
	Females	Males	Tumor	No tumor	Per cent tumor	Non-genic hypothesis	Genic hypothesis
F_1 ♀ × Bagg ♂	6	2	40	5	88.9	Low	High
F_1 ♀ × 1194 ♂	21	5	54	61	47.0	Low	Low
F_1 ♂ × Bagg ♀	12	8	36	13	73.5	High	High

tumor strain than when mated with males from a strain having low tumor incidence. In the third type of mating F_1 males were back-crossed to females from a high-tumor strain. On either hypothesis this type of mating should result in progeny with a high tumor incidence. The terms high and low, applied to expected tumor percentages, are used here in a relative sense and their comparative values would remain the same although the actual level of tumor incidence might be affected by chromosomal or extrachromosomal factors. In the application of the non-genic hypothesis to tumors that are not sex-limited, it must be remembered that males could be affected by the extrachromosomal factor but that these formulae indicate only what they transmit.

The results of crosses with the stocks described, made in accordance with this plan, are shown in Table VIII. All of the parents except one female from the Bagg strain were tarred. All of the F_1 mice used as parents, and all mice from the Bagg strain, except the one that was not tarred, developed tumor. Of the mice belonging to the low-tumor strain, only one had tumor. The number of offspring produced in the three back-crosses ranged from 45 to 115. As stated above, if there is an effective maternal influence, the tumor incidence from the first two crosses should be low. However, the percentages of tumor found were 88.9 and 47.0. Upon the genic hypothesis, without an overbalancing maternal influence, the back-cross to the Bagg strain should give a high incidence and the back-cross to Strain 1194 a lower incidence. Such actually were the results obtained. Mathematically these percentages show significant differences (χ^2 is 23.4 and P is $<.01$). In the third cross the incidence should be high. The group of 49 individuals produced in this type of back-cross showed 73.5 per cent of lung tumor—a figure intermediate between those of the first two cases. This seems somewhat lower than might have been expected on either hypothesis. However, upon comparing it with the highest percentage by means of the χ^2 test for independence, we find that the difference is not clearly significant (χ^2 is 3.60 and P is .06). On the other hand, 73.5 does differ from the lowest percentage to an extent clearly significant (χ^2 is 9.75 and P is $<.01$). It would therefore seem justifiable to class the incidence of tumor obtained in the third type of mating as high. It was noted that in the second type of backcross one parent from the low-tumor strain developed a tumor. If his offspring are omitted from the total, the tumor incidence is 47.2 per cent and the general relationships remain unchanged.

As a whole the data presented here fit the genic hypothesis. Although the strains used for comparison are not 100 per cent positive and negative in regard to susceptibility to lung tumor, the consistency of the data justifies their consideration, and it appears that backcrosses as well as original crosses between differing strains of mice have furnished no evidence of maternal influence in connection with lung tumor.

Most of the percentages of lung tumor shown in the crosses reported here were very high. It might seem at first that these results were due to the conditions of the experiment and that the tumor incidence in all tarred mice would be high. It must be emphasized, however, that the figures for the low strain given in Table I include the results of tarring small groups of animals which were used continuously throughout the experimental period and may therefore serve as controls. Furthermore, the tumor incidence of the offspring resulting from one of the back-crosses was in fact relatively low in that instance in which a lower incidence was prescribed by genetic expectation.

DISCUSSION

It is to be noted that these observations were made upon lung tumor only and obviously have no bearing upon the relation of maternal influence to other types of tumor. We have already expressed the view that in all probability different kinds of tumor are under different control, some being affected more than others by non-genic factors. It might, perhaps, have been expected that lung tumors would not be subject to the same rule as mammary tumors. In the first place, mammary tumors are practically sex limited, while lung tumors may occur in high percentages in both sexes. Maternal influence, however, may be wide-spread. Evidence for it has been presented in connection with mouse leukemia, which is not sex limited, and also in connection with longevity. Since length of life may represent, among other things, resistance to a large number of ordinary laboratory diseases, the phenomenon may not be uncommon. It is important, therefore, that maternal influence has not been found to have a part in the experiments made with respect to lung tumors. While certain environmental factors, such as the cutaneous application of tar or the subcutaneous injection of dibenzanthracene, do have an effect upon the incidence of lung tumor in mice, maternal influence does not seem to play an important rôle, if any.

SUMMARY

Reciprocal crosses were carried out between a strain of mice (No. 1194) having a low incidence of lung tumors and two strains (Bagg derivative and D) with higher incidences. Also three types of back-crosses were made. The incidence of lung tumor in the tarred progeny resulting from these crosses and back-crosses gave no evidence of the factor of maternal influence.

BIBLIOGRAPHY

1. Roscoe B. Jackson Memorial Laboratory: Science 78: 465, 1933.
2. Korteweg, R.: Nederl. Tijdschr. v. Geneesk. 78: 240, 1934.
3. Murray, W. S., and Little, C. C.: Genetics 20: 466, 1935; Science 82: 228, 1935; Am. J. Cancer 27: 516, 1936.
   Bittner, J. J.: J. Heredity 27: 391, 1936; Proc. Soc. Exper. Biol. & Med. 34: 42, 1936.
   Cloudman, A. M., and Little, C. C.: J. Genetics 32: 487, 1936.
4. Korteweg, R., Nederl. Tijdschr. v. Geneesk. 79: 1482, 1935; Genetica 18: 350, 1936.
5. MacDowell, E. C.: Science 81: 418, 1935; Am. J. Cancer 26: 85, 1936.
   MacDowell, E. C., and Richter, M. N.: Arch. Path. 20: 709, 1936.
6. Lynch, C. J.: J. Exper. Med. 43: 339, 1926.

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SEROLOGICAL BEHAVIOR OF HEATED PROTEIN MIXTURES

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Recently it was reported (1) that mixtures of diluted horse and swine sera, heated at 120°C. for 30 minutes, exhibited a new specificity demonstrable by means of immune sera which gave strong reactions with the homologous antigen and only very weak reactions with a mixture of the two sera heated separately. This specificity was also seen in absorption experiments, and it was concluded that the heated mixture "contains one or more specific factors not present in horse serum or swine serum."[1] The development of new specific properties by the procedure described is of such interest that it seemed desirable to study the phenomenon in greater detail.

Preparation of antigens. A mixture of equal volumes of fresh normal horse serum and pig serum was diluted with 3 volumes of distilled water and heated in the autoclave at 120°C. for 30 minutes. Horse serum and pig serum were heated separately in the same way. These three opalescent heated serum solutions were kept sterile in the icebox and used as stock antigens for immunization and the subsequent tests. The dilutions of mixtures are stated in terms of each component, i.e., 1:100 dilution means 1 part horse serum and 1 part pig serum in 100 parts of the solution.

Immunization. Rabbits were immunized with each of the three antigens by giving several courses of injections consisting of daily intravenous doses of 2 cc. of a 1:2 dilution of the serum solutions (1:8 dilution of original serum), with a week's rest after each course. In addition, and in the hope of obtaining more specific sera, one series of rabbits was immunized, according to a method recommended by Wolfe (2), with 3 intravenous injections of 2, 4 and 6 cc. given successively every other day. After one course of such injections, the sera were very weak and a second course was therefore given. Another group of rabbits received 3 intra-abdominal injections of 4 cc. each at weekly intervals.

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[1] It was suggested further that "if analogous fusion of paternal and maternal protein specificities takes place during sexual reproduction, the apparent emergent evolution of one or more new protein characters in certain hybrids would be in accord with Mendelian laws."

TABLE 1

Direct precipitin tests with heated mixture immune sera

The tests were read after 1 hour at room temperature. The intensity of the reactions is indicated as follows: 0, ftr (faint trace), tr (trace), *tr* (strong trace), ±, +̲, +, +±, ++, ++±.

HEATED MIXTURE IMMUNE SERA (2 GTTS.)	ANTIGEN (0.2 cc.)					
	Heated mixture of horse and pig sera (homologous antigen)			Horse and pig sera heated separately (heterologous antigen)		
	1:100	1:500	1:2500	1:100	1:500	1:2500
No. 4-03	+	++	+̲	+	++	+̲
4-04	±	+±	+̲	±	+±	+̲
4-05	+	++	+	+	++	+̲
4-06	++±	+±	±	++±	+±	±
4-18	±	+±	±	±	+±	±
4-20	+̲	+±	0	+̲	+±	0

EXPERIMENTAL

It will be seen from the direct titrations in table 1 that in our experiments the "heated mixture" immune sera reacted with equal intensity on the horse and pig antigens, whether mixed before or after heating, and we were therefore unsuccessful in demonstrating, by direct precipitin tests, the presence of a new specificity in the homologous antigen.

Attempts were then made to demonstrate, as in the paper cited, horse-pig antibodies, differing from horse or pig antibodies, by means of absorption tests. The optimal proportions of heterologous antigen and heated mixture immune serum were first determined by titration. The absorptions with the heterologous antigen (mixture of horse and pig sera heated separately) were carried out by mixing 1 volume of immune serum with ½ volume of the optimal dilution of antigen. These absorption mixtures were incubated at room temperature for from 1 to 2 hours and in the icebox overnight. The absorbed sera were then tested with both homologous and heterologous antigens. The results from 9 different immune sera showed that when the absorption was complete for the heterologous antigen, it was likewise complete for the homologous antigen, i.e. antibodies specific for the horse-pig antigen were not demonstrable.

Attempts were then made to show a difference between the two antigens by means of partial absorption of the immune sera with heterologous antigen. These absorptions were made by mixing 1 volume of immune serum with ½ volume of the antigen dilutions as given in table 2, determined by preliminary titration for optimal proportions. The absorption mixtures were left at room temperature for from 1 to 2 hours, and in the icebox overnight. When the sera were absorbed to varying degrees in this way and tested with both antigens, the heterologous antigen, as a rule, removed antibodies for the homologous and heterologous antigens to the same degree, although in a few instances (e. g., nos. 4–18, 4–21 and 4–22) the absorbed sera reacted slightly stronger with the homologous antigen. Most of these absorption experiments were repeated several times and differences noted between the two antigens are included in the table even though they were not seen regularly on retesting. The experiments with partial absorption were, thus, inconclusive in the demonstration of antibodies specific for the horse-pig antigen.

Although the attempts to demonstrate an antibody specific for the heated serum mixture were, on the whole, unsuccessful, it was considered quite possible that aggregates consisting of both horse and pig proteins might nevertheless be formed in the heated mixture, and an answer to this question was sought in the following experiment. The heated serum mixture was precipitated (in optimal proportions) with an equal volume of precipitin for heated pig serum and the supernatant antigen tested with precipitin for heated horse serum. Another sample of the

TABLE 2

Precipitin tests with partially absorbed heated mixture immune sera

(The tests were read after standing in the icebox overnight)

HEATED MIXTURE IMMUNE SERA ABSORBED WITH HETEROLOGOUS ANTIGEN (0.13 cc.)	DILUTION OF HETEROLOGOUS ANTIGEN USED* FOR ABSORPTION	ANTIGEN (0.2 cc.)	
		Heated mixture (homologous antigen) 1:500	* Horse and pig sera heated separately (heterologous antigen) 1:500
No. 4-03	1:50	0	0
	1:200	±	±
	1:800	++	++
4-04	1:100	0	0
	1:400	*tr*	tr
	1:1600	+±	+±
4-05	1:50	0	0
	1:200	±	±
	1:800	++	++
4-06	1:50	0	0
	1:200	+±	+±
	1:800	++±	++±
4-18	1:100	0	0
	1:400	tr	0
	1:1600	±	±
4-19	1:50	0	0
	1:200	+±	+±
	1:800	++	++
4-20	1:50	0	0
	1:200	±	±
	1:800	+±	+±
4-21	1:100	0	0
	1:400	tr	ftr
	1:1600	+	±
4-22	1:200	0	0
	1:800	tr	ftr

TABLE 3

Tests for the presence, in heated mixture antigen, of aggregates consisting of both horse and pig serum proteins

The readings in the first line were taken after 15 minutes at room temperature and those in the second line after overnight in the icebox

A. (*a*) 0.8 cc. precipitin no. 4-07 for heated pig serum + 0.8 cc. heated mixture antigen, diluted 1:200, as determined by preliminary titration for optimal proportions.

(*b*) 0.8 cc. precipitin no. 4-11 for heated horse serum + 0.8 cc. heated mixture antigen, diluted 1:200.

(*a*) and (*b*) were left at room temperature 1 hour and in the icebox overnight. The centrifuged supernatant of (*a*) was then tested with precipitin for heated horse serum and that of (*b*) was tested with precipitin for heated pig serum.

Control: 0.8 cc. saline + 0.8 cc. heated mixture antigen, diluted 1:200 as in (*a*) and (*b*).

(2 GTTS.)	A. (*a*) SUPERNATANT (0.2 CC.)						CONTROL (0.2 CC.)					
	Undiluted	1:2	1:4	1:8	1:16	1:32	Undiluted	1:2	1:	1:8	1:16	1:32
Precipitin no. 4-11 for heated horse serum	0 ±	0 ±	0 *tr*	0 tr	0 ftr	0 0	+± ++	+ +±	± +	tr ±	ftr tr	0 ftr
	A. (*b*) SUPERNATANT											
Precipitin no. 4-07 for heated pig serum	0 +	0 ±	0 ±	0 *tr*	0 ftr	0 0	+± ++±	+± +±	+ +	tr ±	0 tr	0 ftr

B. Control experiments to test for cross reactions were performed as above except that as antigen a mixture of horse and pig sera, heated separately, was used.

	B. (*a*) SUPERNATANT											
Precipitin no. 4-11 for heated horse serum	+ ++±	+ +±	± +	tr +	ftr ±	0 tr	+± ++±	+ +±	± +	*tr* +	ftr ±	0 tr
	B. (*b*) SUPERNATANT											
Precipitin no. 4-07 for heated pig serum	+± ++	+ ++	± +±	± +	ftr *tr*	0 tr	++ ++	+ ++	± +	± ±	ftr ±	0 tr

same antigen was similarly treated with precipitin for heated horse serum and the supernatant antigen subsequently tested with precipitin for heated pig serum. It will be seen from the results in table 3 that precipitation of the heated mixture antigen with precipitin for either heated pig or horse serum reduced considerably the reaction with the precipitin of the other species, which, if not due to cross reactions, is difficult to explain otherwise than on the assumption of the presence of aggregates composed of both species proteins.

That the phenomenon observed cannot be attributed to cross reactions was shown by control experiments in which a mixture of pig and horse sera heated separately was used. In this case (table 3B) each immune serum precipitated out of the mixture only its homologous antigen leaving the heterologous antigen practically undiminished.

The various heated antigens used in these experiments were all equally opalescent and the protein aggregates formed as the result of heating were shown to be of sufficiently large size to be partially sedimented in the centrifuge at 4500 r.p.m. After centrifuging for 2 hours, respective samples of each showed top layers which were almost clear, intermediate opalescent layers and lower yellow viscous layers. It was found on titration with precipitin sera that the bottom layer of the centrifuged antigen was about 16 times more concentrated than the top layer.

SUMMARY

It may be concluded from tests with precipitin for either species component that, on heating a mixture of proteins from two animal species, aggregates are formed which consist of the two sorts of protein. However, the appearance of new specific properties in these aggregates could not be conclusively demonstrated.

REFERENCES

(1) Zoet, August G., Proc. Soc. Exp. Biol. and Med., 1935, 32, 1469.
(2) Wolfe, Harold R., Jour. Immunol., 1935, 29, 1.

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STUDIES ON THE DIFFUSION EFFECT UPON IONIC DISTRIBUTION

II. EXPERIMENTS ON IONIC ACCUMULATION

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The present report furnishes experimental confirmation of the first paper of this series (1), which gave a theory for the ionic distribution in the steady state of a system of a type that may well occur in biology (*cf.* Fig. 1).

Review of the Theory

The system considered and the theoretical results are recapitulated as follows:

Across a permeable boundary *m* there was supposed to be present a constant difference in concentration of either the cation D^+ or the anion A^-. The maintenance of this condition, accomplished, for example, by a continuous addition of the substance *DA* to the rather small volume (*i*), causes *DA* to act as a "diffusion agent" which steadily diffuses across *m* into the part (*o*). In (*o*) a fixed, constant composition was maintained by keeping the volume large.

It was predicted that the continuous steady diffusion of *DA* was bound to influence the distribution of other electrolytes present, denoted by $M'B'$; $M''B''$ etc., which were not participating in any active diffusion as was *DA*. These ions were called "passive ions." If, for example, the initial composition was the one graphically shown in Fig. 2 *a*, the ultimate effect of the steady diffusion of *DA* would consist in an accumulation of M^+ ions and an impoverishment of B^- ions on the inside, as pictured in Fig. 2 *b*. It was supposed that the D^+ ions had a higher mobility than the A^- ions.

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* The experiments were carried out when the author held a Rockefeller Foundation Fellowship.

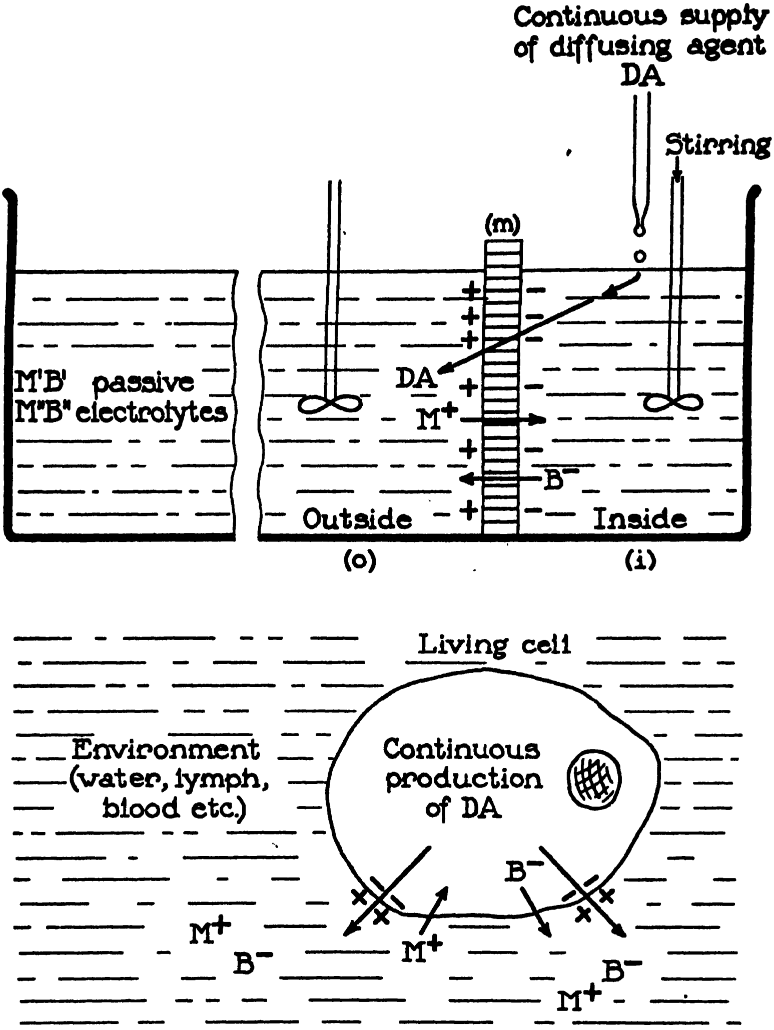
Continuous supply of diffusing agent
DA
Stirring
(m)
o
M'B' passive
M"B" electrolytes
DA
M+
B−
Outside
Inside
(o)
(i)
Living cell
Environment (water, lymph, blood etc.)
Continuous production of DA
M+
B−
M+
B−
B−
M+

FIG. 1. Scheme of the system considered. The upper part shows the principle of the experimental arrangement. The lower part demonstrates schematically a biological analogy to this system.

The theory leads to the following alternative expressions for the distribution of the passive ions:

(*a*) In terms of the final electrical (diffusion) potential π, for univalent ions,

$$\log \frac{M_i^+}{M_o^+} = \log \frac{B_o^-}{B_i^-} = \frac{F}{RT} \cdot \pi \tag{1}$$

where M_i^+, B_i^-, and M_o^+, B_o^-, denote concentration inside and outside respectively, F is the Faraday, R is the gas constant, and T the absolute temperature. When π is expressed in millivolts, F/RT is equal to 1/58 (at 18°C.).

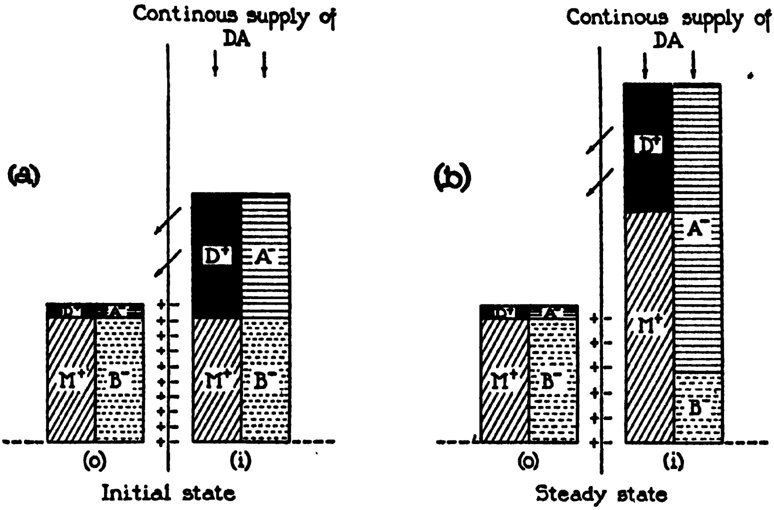

FIG. 2. The effect of a steady diffusion of DA upon the ionic distribution when $u_D > v_A$. DA is by some means (by addition or production) steadily supplied inside (i). The concentrations of the electrolytes outside (o) are kept constant. An electrical potential arises across the boundary (membrane). This causes an inward migration of M^+ and an outward migration of B^-. Finally the concentration gradients have become sufficiently large to balance the electrical gradient and the system approaches a steady state. Thus the M ions became accumulated and the B ions diminished in amount inside.

The scale used in this figure corresponds very closely with the conditions of Experiment 1 (see Table I).

(*b*) In terms of known concentrations the distribution can be explicitly calculated from the following transcendental equation

$$\log \xi = \log \frac{M_i^+}{M_o^+} = \log \frac{B_o^-}{B_i^-} = \frac{u_D - v_A}{u_D + v_A} \log \frac{D_i^+ + \Sigma M_i^+}{D_o^+ + \Sigma M_o^+} \tag{2}$$

Here u_D and v_A denote the mobility of the D^+ and A^- ions in the diffusion layer. It should be noticed that $(D^+ + \Sigma M^+)$ is equal to the *total* concentration of the electrolytes.

Furthermore, the fact was brought forward, as is evident from Equations 1 and 2 here, that the relationship

$$\frac{M_i'^+}{M_o'^+} = \frac{M_i''^+}{M_o''^+} = \cdots = \frac{B_o'^-}{B_i'^-} = \frac{B_o''^-}{B_i''^-} = \cdots = \xi \tag{3}$$

must be valid. Equation 3 is immediately recognized as being characteristic also for the Gibbs-Donnan equilibrium, which obviously constitutes a special case of the more general system described by Equation 2, obtained when u_D or v_A approaches zero.

It should be emphasized that neither Equation 1 nor Equation 3 alone permits any explicit calculation of the ionic distribution. The factors which determine the distribution picture are: (*a*) The mobility relation u_D/v_A, (*b*) the concentration ratio $D_i^+/\Sigma M_o^+$, and, (*c*) the concentration ratio D_i^+/D_o^+.

The further from unity u_D/v_A is and the higher $D_i^+/\Sigma M_o^+$ or D_i^+/D_o^+ is, the higher accumulation (or impoverishment) will be attained by the passive ions. This statement follows from Equation 2. In a less abstract way, it can also be expressed with the aid of Equation 1 by saying that the greater diffusion potential present across the boundary, the more marked accumulation effects (or the reverse) are obtained.

Finally it should be emphasized that this diffusion effect upon ionic distribution is *not* any kind of cataphoresis effect, because no current is flowing. Although interpretations in terms of a (diffusion) potential are rather convenient, it might be more correct to employ a concept of ionic exchange.

Earlier Experiments

The phenomenon of accumulation and impoverishment of certain ions in a system where a steady diffusion takes place is related to a great many experimental observations.

These are described in the literature as "*Diffusion gegen das Konzentrationgefälle*" by Behn (2) in 1897; "*Diffusion retrograde*" by Thovert (3) in 1902; "Counterdiffusion" by Osborne and Jackson (4) in 1914; and Walpole (5) in 1915, etc. These workers simply placed two solutions on top of one another, one containing the diffusion agent, both having another electrolyte in common. After an arbitrary time two or more parts of the diffusion column were analyzed. In general a redistribution of all ions could be observed. Apparently there was no possibility of obtaining any steady state with these conditions. The effects shown ought correctly to be classified as "temporary diffusion effects" (see (1) p. 155) and were in most cases rather small. In the best cases the differences of passive ion concentration amounted to from 15 to 20 per cent. Some experiments with conditions corresponding to the theory outlined above and of the same type as will be reported here, were carried out some years ago by Straub (6). He observed an accumulation of cations and a simultaneous impoverishment of anions in a clay cylinder, inside of which acid was supplied. Straub failed, however, to offer any satisfactory discussion of what he called the "*Harmonie-Einstellung*" of the ions, nor did his data seem to be suitable for any treatment in regard to the theory now accessible.

This subject is also related to the numerous cases of "accelerated or retarded diffusion" of electrolytes reported, for instance by McBain and Dawson (7) (*cf.* also reference (8)). This is quite natural, because the theoretical considerations here advanced are founded on the basis of Nernst's (9) classical kinetical theory for electrolyte diffusion.

Summarizing the previously published works, it may be said, that there were no experiments available for use as a test for the theory proposed for a steady state.

EXPERIMENTAL PROCEDURE

In principle the set up was that sketched in Fig. 1: a small "inside volume," 10–30 cc., was separated from a large "outside" volume (0.5–1.5 liters) by a porous convection-proof membrane, *m*. Both solutions were kept homogeneous up to the membrane by stirring. In (*i*) the diffusing agent was continuously supplied either in the form of a slow stream of a concentrated solution ($HClO_4$), or by addition of the agent in a solid form so as to maintain a saturated solution (KIO_3).

Apparatus.—The apparatus used has been described in another paper (10). The supply of the concentrated diffusing agent in Experiment 1, $HClO_4$, was regulated by varying the level of a Mariotte's vessel, which, through a flexible tube ending in a long capillary jet, was in communication with the inside solution. Only one membrane was employed, consisting of four superimposed layers of cellophane 600 (Du Pont Company), in wet condition of a total thickness of 0.28 mm. With one or two layers the results showed great divergences from the theoretically expected. This circumstance is to be ascribed to the influence of "unstirred layers" adhering to the membrane surfaces. These extra diffusion layers are not convection-proof. By means of a special method their thickness is estimated to be approximately 0.03 mm. The cellophane may have a slight influence upon the mobilities of the ions here investigated, which, however, will be accounted for when comparing the results with the theory.

Choice of Electrolytes.—This was dictated partly by a desire to produce pronounced distribution differences, partly by attention to available analytical methods. Therefore, the first experiment to be reported was carried out with an acid as a diffusing agent and with NH_4Cl as source of the passive ions (u_H/v_{ClO_4} being large (*cf.* p. 256), H_4N and Cl are easily determined). In the second experiment a neutral salt, K-iodate, was used as a diffusing agent, because the iodate ions have a rather low mobility compared with K ($u_K = 65$, $v_{IO_3} = 33$). Hydrochloric acid furnished the passive ions.

Analyses.—The analyses were performed with micro methods. The samples taken had a volume ranging from 0.05 to 1 cc. depending upon the circumstances. H was determined by titration with 0.01 N NaOH using methyl red as indicator. Cl was determined electrometrically after the acidity titration, employing 0.005 N $AgNO_3$ and sulfuric acid. NH_4 was analyzed iodometrically with 0.05 NaBrO

TABLE I

Development of Ionic Accumulation and Impoverishment in a System Subjected to a Steady Diffusion

Experiment 1

Initial state
0.1 N $HClO_4$
(10 cc.) | 0.1 N H_4NCl (1500 cc.)
0.1 N H_4NCl
Inside (*i*) | Outside (*o*)
(Membrane)

H_i maintained constant by continuous addition of 5 N $HClO_4$.

Time	Inside (*i*)				Outside (*o*)				$\frac{(H_4N)_i}{(H_4N)_o}$	$\frac{(Cl)_o}{(Cl)_i}$
	H	H_4N	Cl	ClO_4*	H	H_4N	Cl	ClO_4*		
hrs.	*mM./l*	*mM./l*	*mM./l*	*mM./l*	*mM./l*	*mM./l*	*mM./l*	*mM./l*		
0	90.9	99.1*	99.1	90.9	0	99.9*	99.9	0	1.00	1.01
0.5	84.1	111	89.0	106	—	—	—	—	1.11*	1.12
2	96.5	159	62.1	193	2.95	99.9	100.9	2.0	1.59	1.62
4	99.6	188	51.6	236	4.31	99.1	101.8	(1.6)	1.90	1.97
6	99.6	207	48.6	258	5.85	102	102	5.9	2.03	2.10
7	96.5	212	48.6	260	—	—	—	—	2.08*	2.10*
8	111	215	47.9	278	8.00	102	101.4	8.6	2.11	2.12
9	105	214	48.6	271	8.40	101	101.5	7.9	2.13	2.09
9^{20}	Potential (*i*)–(*o*): Calomel electrodes + 21.0 mv.; Ag/AgCl − 0.8 mv.†									
20	121	214	49.1	286	17.7	101	100	18.7	2.13	2.04
20^{30}	Potential (*i*)–(*o*): Calomel electrodes + 20.2 mv.; Ag/AgCl − 1.2 mv.†									

* Calculated figures.

† Sign outside.

and 0.01 N $Na_2S_2O_3$ according to (13). IO_3 was titrated as I_2 with thiosulfate after addition of KI and acid. Other concentrations were calculated by difference. All pipettes, burettes, and solutions were calibrated against standard solutions. No analytical error is likely to exceed ± 2 per cent.

Potential Measurements.—The potential measurements were performed by means of calomel electrodes, as described elsewhere (11). Supplementary measurements were made with Ag/AgCl electrodes prepared according to Brown (14). The electrodes were connected either to a push pull vacuum tube voltmeter, or to a Leeds-Northrup Type K potentiometer. The potential figures are regarded as accurate to ± 0.5 mv.

TABLE II

Development of Ionic Accumulation and Impoverishment in a System Subjected to a Steady Diffusion

Experiment 2

Initial state
(10 cc.) *ca.* 0.4 N KIO_3 saturated plus crystals | 0.01 N HCl (500 cc.)
Inside (*i*) | Outside (*o*)
(Membrane)

Time	Inside (*i*)				Outside (*o*)*				$\frac{(H)_i}{(H)_o}$	$\frac{(Cl)_o}{(Cl)_i}$
	H	K†	Cl	IO_3	H	K†	Cl	IO_3		
hrs.	*mM./l*	*mM./l*	*mM./l*	*mM./l*	*mM./l*	*mM./l*	*mM./l*	*mM./l*		
0	0	—	—	—	10.0	—	—	—	—	—
0.1	1.7	371	0.9	372	10.0	0.4	10.0	0.4	0.17	11.1
0.5	10.3	—	—	336	10.0	1.6	10.5	1.1	1.03	—
1	21.0	327	2.9	345	9.8	1.7	9.7	1.8	2.14	3.34
2	37.4	333	4.0	366	—	—	—	2.7	(3.82)	—
3	47.5	328	4.6	376	9.2	4.5	9.9	3.8	5.16	2.15
4	56.0	311	5.0	362	10.2	1.2	9.8	1.6	5.50	1.60
5	62.1	318	5.1	375	10.1	2.5	9.5	2.5	6.16	1.86
6	63.0	315	5.3	383	9.8	4.0	10.1	3.8	6.44	1.90
7	63.0	303	5.2	366	10.9	1.9	10.2	2.6	5.78	1.96
9	61.0	271	5.6	326	10.2	3.3	10.3	3.3	5.98	1.84
10	57.1	248	5.6	300‡	—	—	—	—	—	—
9^{30}	Potential (*i*)–(*o*): Calomel electrodes + 25.5 mv.§									

* The solution was renewed after 3 and 6 hrs., thereby restoring the initial composition.

† Calculated values.

‡ The decrease in KIO_3 concentration after 6 hrs. was probably due to a fall of the room temperature, hence decrease of solubility.

§ Sign outside.

The pH and Cl activity measurements were made by means of a glass electrode (type MacInnes and Dole) (18), and a Ag/AgCl electrode (Brown (14)) respectively, using a saturated calomel electrode as reference. A Compton electrometer served as zero instrument.

RESULTS AND DISCUSSION

In Tables I and II two typical experiments are recorded (Experiments 1 and 2).

Experiment 1.—This was designed to fulfill, as completely as possible, the main assumptions on which the theory was based: (*a*) Uni-univalent electrolytes being present only, all known as strong and free from complicating phenomena; (*b*) no migration of water across the convection-proof boundary. Perchloric acid served as diffusing agent and the passive ions were H_4N and Cl. The $HClO_4$ concentration in the small inside chamber was maintained at about 1/10 molarity by means of an external supply. The experiment was started with equal concentration of H_4N and Cl ions inside and outside. During the following time, however, one could observe that the H_4N ions moved inwards and, simultaneously, Cl ions moved outwards. Finally a steady state was approached inside, resulting in a 2.13 fold accumulation of H_4N and a Cl impoverishment of almost the same magnitude. Fig. 2 corresponds very closely with the conditions of this experiment. No appreciable water migration could be detected.

The experimental results will now be compared with a theory: The theoretical distribution ratio

$$\xi = \frac{(\text{Passive cation concentration})_i}{(\text{Passive cation concentration})_o} = \frac{(\text{passive anion concentration})_o}{(\text{passive anion concentration})_i}$$

can be calculated from either of Equations 1 or 2.

According to Equation 1, at 20°C. and with the diffusion potential expressed in millivolts,

$$\pi = 58.1 \log \xi \tag{3}$$

In the experiment the membrane potential π, was + 20.2 mv., hence, the distribution ratio is

$$\xi = 2.23, \text{ calculated from observed } \pi$$

(Observed value for H_4N is 2.13, for Cl is 2.04 after 20 hours.)

According to Equation 2 combined with the relation $\xi = \frac{(H_4N)_i}{(H_4N)_o}$

$$\log \xi = \frac{u_H - v_{ClO_4}}{u_H + v_{ClO_4}} \log \frac{(H)_i + \xi \cdot (H_4N)_o}{(H)_o + (H_4N)_o} \tag{4}$$

or
$$\log \xi = q \cdot \log \frac{121 + \xi \cdot 101}{17.7 + 101} \tag{4a}$$

where q stands for $(u - v)/(u + v)$. There are two possibilities of choosing q, either to use q as valid for "free" water, or, to use q as determined for cellophane. Employing the mobilities $u_H = 315$ and $v_{ClO_4} = 64$ as given for free water in the Landolt-Börnstein Tables (15), q becomes 0.66. The relative mobility in cellophane was determined from measurements of the diffusion potentials between 0.1 N and 0.01 N $HClO_4$ by means of a procedure described elsewhere (11). The mobility ratio, u/v, thus obtained was 7.55[1] and q for cellophane was calculated to 0.767. Alternately inserting these q values in the Equation 4 *a*, the following values of ξ are obtained

$\xi = 1.89$, calculated from Equation 4 when $q = 0.66$ (H_2O)
$\xi = 2.30$, " " " " " $q = 0.767$ (cellophane)

Corrections for Activity Changes.—All discussions so far have been carried out on the supposition that the activity coefficients, γ, were all 1.00. Of course, this is not true when dealing with rather concentrated solutions as is the case in these experiments. Perhaps it may be somewhat meaningless to attempt any adjustments of the experimental results with regard to the activities, because the activity concept was not employed in the theory to be tested. Nevertheless, it may be interesting to introduce some activity corrections. Although, the procedure to be used is only approximate, one should get an idea as to the direction in which the results are influenced by the interionic forces.

The observed distribution ratios obtained from *concentration* figures may be transformed into ratios between activities by multiplying through with appropriate coefficients. Attention will be paid only to the H_4N distribution. The H_4N ions were paired on both sides of the membrane with chloride or perchlorate. It seems justifiable to assume that the mean activity coefficients of the two salts are rather similar to those of KCl. Secondly, the usual supposition is made, that γ for an electrolyte component in a mixture is the same as in a solution containing only this electrolyte at an ionic strength corresponding to that of the mixture (the rule of Lewis and Randall, *cf.* for instance Falkenhagen (16), p. 71). Thus γ for the approximate 0.3 N inside solution was taken as 0.67, and for the approximate 0.1 N outside as 0.75 (according to figures for KCl given by MacInnes and Noyes (17)). Introducing these values, we obtain

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[1] u/v for free water is $315/64 = 4.94$, hence the relative cation mobility is about 50 per cent higher in cellophane. For several chlorides the corresponding increase was determined as about 40 per cent (Teorell (11)). A similar relation was observed also for KIO_3 (see footnote 4).

$$\text{Observed } \xi \text{ corrected for activity} = \left(\frac{0.67}{0.75} \cdot 2.13\right) = 1.91.$$

Corresponding activity corrections may also be applied to Equation 4 *a*, (γ for $HClO_4$ was taken as 0.78 and 0.82 inside and outside respectively). The results are

$\xi = 1.54$, calculated from Equation 4 and corrected for activity when $q = 0.66$ (H_2O)
$\xi = 1.70$, " " " " " " " " " $q = 0.767$ (cellophane)

Experiment 2.—The experimental conditions were not quite ideal, because certain ionic interactions took place, and water migration also occurred. These factors were not considered in the theory. Therefore, this experiment is an interesting one, giving some information as to the sensitiveness of the proposed theory towards new factors. The diffusing agent was potassium iodate, being in a saturated solution inside and kept in contact with an excess of KIO_3 crystals. The passive ions were formed from hydrochloric acid, at the beginning of the experiment being present on the outside only. During the course of the experiment the ionic concentration outside remained practically constant because of its large volume and repeated renewals. In the inside chamber, however, a redistribution of the electrolytes was developed resulting in a steady state. Here, the passive cations, in this case the H ions, were accumulated.[2] The passive anions, the Cl anions, in the final steady state attained a considerably lower concentration inside than outside (Table II).

Qualitatively the observed diffusion effect upon the ionic distribution is in agreement with the theory. With the direction the diffusion potential has, the negative sign being inside, an accumulation of passive cations and impoverishment of anions is to be expected, and was also found. As to the quantitative relations this experiment, at first, seems to show some discrepancies between theory and observations.

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[2] The increase of C_H inside could be nicely demonstrated, if more dilute HCl was used, by the color changes of an added indicator such as brom phenol blue.

TABLE III

Hydrogen Ion Activity Measurements of Inside and Outside Solutions after 9 Hours

Experiment 2

(P.D. measured with glass electrode/calomel electrode, "slope" 57.5 mv.)

Solution	Total H concentration	Millivolts	pH	a_H
	mM./l			*mM./l*
Inside	61.0	−18.6	1.61	38.9
Outside	10.2	+5.1	2.02	9.55
0.01 N HCl (control)	10.0	+5.6	2.03	9.34
(*i*)–(*o*)	50.8	−23.7	−0.41	$\frac{(a_H)_i}{(a_H)_o} = 2.57$

TABLE IV

Chloride Ion Activity Measurements of Inside and Outside Solutions after 9 Hours

Experiment 2

(P.D. measured with Ag/AgCl electrode/calomel electrode, "slope" 57.7 mv.)

Solution	Total Cl concentration	Millivolts	pCl	a_{Cl}
	mM./l			*mM./l*
Inside	5.6	−118.8	2.37	4.20
Outside	10.3	−96.5	1.98	10.2
0.01 N HCl (control)	10.0	−98.9	2.03	9.34
(*i*)–(*o*)	−4.7	−22.3	0.39	$\frac{(a_{Cl})_o}{(a_{Cl})_i} = 2.43$

However, when due attention is paid to certain interfering circumstances, the results also appear to agree quantitatively in a satisfactory manner with the theory. For this purpose the following discussion is given.

First, in Table II it will be noticed that the final distribution ratio

H_i/H_o is as high as 5.98; the ratio Cl_o/Cl_i, however, is much lower, 1.84. The H figures were determined by titration, accordingly all hydrogen ions, both free and bound, were included. It is well known that IO_3 ions can form $H(IO_3)_2$, bi-iodate, in *acid* solution, thus binding hydrogen ions. Unfortunately, pH measurements were not performed during the actual run, but afterwards "artificial" inside and outside solutions were made up to correspond with the analytical figures recorded in Table II at 9 hours.[3] In these solutions pH and a_{Cl} were determined by means of the glass electrode and AgCl electrode respectively. The results are summarized in Tables III and IV. It can be seen that the H ion activity inside is considerably lower than the total H concentration, as pointed out, probably due to formation of bi-iodate ions. From these measurements the ionic distribution ratios, expressed in activity figures, will be found as

Observed $\xi = 2.57$ (5.98), corrected for H activity
" $\xi = 2.43$ (1.84), " " Cl "

The values within brackets are the ones obtained from total concentration figures.

Hence, the relation $H_i/H_o = Cl_o/Cl_i$ seems to be obeyed, as demanded by the theory. The theoretical ξ values are calculated, as in Experiment 1, either from the observed diffusion potential (Equation 1), or from the transcendental formula (Equation 2). For the first alternative is obtained

$\xi = 2.75$, calculated from the diffusion potential

Calculations on the basis of Equation 2 can be performed in several different ways. First, there are two choices of the relation u/v or of q, referring to free water or to cellophane. Secondly, when dealing with a rather concentrated salt solution (saturated KIO_3), some kind of correction for activity changes may be demanded. Based on concentration figures given for 9 hours in Table II it is found that

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[3] These "artificial" solutions were made: *inside*, 5.6 cc. 0.1 N HCl, 55.4 cc. 0.1 N HIO_3, 5.79 gm. KIO_3: *outside*, 10.2 cc. 0.1 N HCl, 0.1 cc. 0.1 N KCl, 3.2 cc. 0.1 N KIO_3; in all cases H_2O was added to make 100 cc. of solution. The reagents were Kahlbaum brands; the solutions were freshly made and standardized.

$\xi = 2.86$, calculated from Equation 2 when $q = 0.327$ (H_2O)[4]
$\xi = 5.14$, " " " " when $q = 0.509$ (cellophane)

Corrections for Activity Changes.—When attempting activity corrections, it was assumed that the mean activity coefficient, γ, for the K-iodate inside was the same as for 0.3N KCl being 0.67. γ_{HCl} outside was taken as 0.93, a figure valid for 0.01 N HCl (according to MacInnes and Noyes). Any further corrections do not have any appreciable effect upon the results and must also be rather ambiguous. Therefore they are omitted. The ξ values obtained from activity figures were

$\xi = 2.55$, calculated from Equation 2 and corrected for activity when $q = 0.327$ (H_2O)
$\xi = 4.25$, " " " " " " " " when $q = 0.509$ (cellophane)

TABLE V

Comparison between Observed and Theoretical Distribution Ratios (ξ) *of Passive Ions in Steady State*

$$\xi = \frac{(\text{Passive cation})\ \text{inside}}{(\text{Passive cation})\ \text{outside}} = \frac{(\text{passive anion})\ \text{outside}}{(\text{passive anion})\ \text{inside}}$$

Experiment	Diffusing agent	Passive ion	Observed from		Calculated from Equation 2 (4)				Calculated from diffusion potential
					Free water		Cellophane		
			Concentration	Activity	Concentration	Activity	Concentration	Activity	
1	$HClO_4$	H_4N	2.13	1.91	1.89	1.54	2.30	1.70	2.23
		Cl	2.04	(1.87)					
2	KIO_3	H	5.98	2.57	2.86	2.55	5.14	4.25	2.75
		Cl	1.84	2.43					

SUMMARY OF RESULTS AND CONCLUSIONS

In Table V the experimental and theoretical results are put together. The figures seem to justify the following conclusions:

1. The agreement between observed and calculated ξ values is, on the whole, quite satisfactory in both the experiments, if proper attention is paid to activity conditions. ξ as calculated from the transcendental

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[4] Calculated value from u_K/v_{IO_3} determined from diffusion potential across cellophane of 0.1 – 0.01 N KIO_3 according to (11). This value was found to be 3.08 (in free water 65/33 = 1.98).

Equations 2 or 4, using the ionic mobilities for free water, yields the best agreement.

2. If the mobility relations as determined for cellophane are used in the theoretical calculations, much higher ξ values than those actually observed would be expected in the KIO_3 experiment. In the $HClO_4$ experiment it is hard to point out any significant difference as to the outcome of different choice of mobility relations. Hence, it appears that it would be better to assume a validity of free water ionic mobilities, in spite of the fact that the diffusion effects occurred across cellophane. Such a conclusion also follows from the views expressed by the author, that the cellophane mobility figures as determined are only *apparent* values. The reason is to be sought in interference from the membrane itself (Teorell (12)).

3. The theoretically expected ξ values when calculated from the *measured* diffusion potential are of the same magnitudes as the observed figures corrected for activity. The latter are only 14 and 9 per cent lower than the former, in Experiments 1 and 2 respectively; these differences may very likely arise from small, unavoidable liquid junction potentials at the calomel electrodes. This fact seems to offer an ample confirmation of one consequence of the theory, namely, that the distribution of the passive ions is completely determined by the *observed* diffusion or membrane potential according to the simple Equation 1, or

$$\log \xi = \frac{\pi}{0.1983\,T} \qquad (1)$$

(π = millivolts; T = absolute temperature.)

In regard to the thermodynamical interpretation of this equation we may refer to the first paper in this series (1), paragraphs IV*b* and *c*.

4. The agreement between experiment and theory according to Equation 1 is of a profound and essential significance. This shows that the water migration does not have any appreciable influence upon the expected ionic distribution, although amounting to about 40 per cent as volume increase inside in Experiment 2. Furthermore, the high ξ values as calculated from cellophane mobilities in Experiment 2 can be ruled out as being improbable, because such figures would correspond to a value of the diffusion potential much higher than that

actually observed. This point offers additional evidence for the views expressed under paragraph 2 above.

5. Still, there are some other points which can be advanced giving beautiful confirmation of the proposed theory. In the theoretical paper (1), in paragraph IV*c* it was remarked:

> "... As the diffusion potential π is equal to the expression for the electrode potential for any passive ion, a concentration chain consisting of reversible electrodes for such an ion species placed in the solutions (*o*) and (*i*) should give no current, ..."

In Experiment 1 the actual "concentration chain potential" with AgCl electrodes, reversible in regard to the passive chloride ions, was measured from − 0.8 to − 1.2 mv. The theoretical value should be ± 0 mv., accordingly the result is not far outside the experimental limit of error. At the same time π was about + 21 mv. (cf. Table I).

In Experiment 2 the same thing can be shown somewhat more indirectly. Here reversible electrodes for both the passive ion species were available, the glass electrode for the H ions, and the AgCl electrode for the Cl ions. The "electrode potentials" for these two ion species are simply found as differences between the potential values recorded in Tables III and IV, as measured against the same reference (calomel) electrode. These values are (*cf.* also the tables),

$$\begin{array}{l}\text{Electrode}\\ \text{potentials}\end{array}\begin{cases}[(\text{mv.})_i - (\text{mv.})_o]_{\text{glass}/\text{Hg}_2\text{Cl}_2} \;\ldots\ldots\ldots\; -23.7 \text{ mv.}\\ [(\text{mv.})_i - (\text{mv.})_o]_{\text{AgCl}/\text{Hg}_2\text{Cl}_2} \;\ldots\ldots\ldots\; -22.3 \text{ mv.}\end{cases}$$

Diffusion potential observed, π, ... +25.5 mv.

It is noticed that the figure for the diffusion potential is almost equal to the figures for the practically identical electrode potentials. As it carries opposite sign, the resulting concentration chain potentials, being the sum of both (diffusion potential + electrode potential), also in this case, will not be far from zero.

SUMMARY

The aim of this paper is to present confirmation of the theory of the diffusion effect. By diffusion effect is understood a redistribution of the ions in a system where a continuous diffusion of one electrolyte is going on, which results in a steady state showing accumulation and impoverishment of certain ions.

Some typical experiments are recorded and discussed. The results are found in satisfactory agreement with the theory: this can be demonstrated in several ways.

The importance of considering activity changes is pointed out. Water migration and the diffusion membrane probably have no appreciable influence upon the diffusion effect under the conditions of these experiments.

The diffusion effect may have biological analogies.

BIBLIOGRAPHY

1. Teorell, T., *Proc. Nat. Acad. Sc.*, 1935, **21**, 152.
2. Behn, U., *Wied. Ann.*, 1897, **62**, 54.
3. Thovert, J., *Compt. rend. Acad. sc.*, 1902, **134**, 826.
4. Osborne, W. A., and Jackson, L. C., *Biochem. J.*, London, 1914, **8**, 246.
5. Walpole, G. S., *Biochem. J.*, London, 1915, **9**, 132.
6. Straub, J., *Kolloid-Z.*, 1933, **62**, 13; 1933, **64**, 72.
7. McBain, J. W., and Dawson, C. R., *J. Am. Chem. Soc.*, 1934, **56**, 52.
8. *Tr. Faraday Soc.*, 1935, **31**, several papers on diffusion.
9. Nernst, W., *Z. phys. Chem.*, 1888, **2**, 617; 1889, **4**, 154. *Cf.* also Planck, M., *Wied. Ann.*, 1890, **40**, 561.
10. Teorell, T., *J. Biol. Chem.*, 1936, **113**, 735.
11. Teorell, T., *J. Gen. Physiol.*, 1935–36, **19**, 917.
12. Teorell, T., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33**, 382.
13. Teorell, T., *Acta med. Scand.*, 1928, **68**, 305.
14. Brown, A. S., *J. Am. Chem. Soc.*, 1934, **56**, 646.
15. Landolt, H., and Börnstein, R., Physikalisch-chemische Tabellen, Berlin, Julius Springer, 5th edition (Roth, W. A., and Scheel, K.), 1923, **1**, 170.
16. Falkenhagen, H., Die Elektrolyte, Leipsic, S. Hirzel, 1932.
17. MacInnes, D. A., and Noyes, A. A., *J. Am. Chem. Soc.*, 1920, **42**, 239.
18. MacInnes, D. A., and Dole, M., *Ind. and Eng. Chem., Analytical Edition*, 1929, **1**, 57.

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THE SEDIMENTATION CONSTANT OF OVOVERDIN

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Through the courtesy of Dr. K. G. Stern and Dr. K. Salomon and with their help, preliminary ultracentrifugal studies have been made of rates of molecular sedimentation in ovoverdin solutions prepared from lobster eggs according to the method described in the preceding note.[1] Several points of interest concerning the degree of purity of such solutions and the order of molecular size and homogeneity of the pigmented protein they contain are brought out in this way.

These studies of sedimentation rates are based on series of photographs made with an analytical air-driven ultracentrifuge[2] arranged for measurements according to the original absorption method of Svedberg. Exploratory examination of a solution of the protein in half-saturated ammonium sulfate indicated the presence of a homogeneous protein so light that it should be sedimented from a solvent of lower density. All subsequent measurements were accordingly made, using as starting material a solution freshly dialyzed to remove all but a trace of the ammonium sulfate.

The pigmented solutions of ovoverdin are dark green in color. They show[1] a very strong light absorption in the blue and violet and an appreciable absorption in the yellow; besides this they have the usual protein ultra-violet absorption of wave-lengths below ca. 2800A. Series of sedimentation photographs have been made with blue light and with ultra-violet light shorter than 2700A; simultaneous visual observations were made with yellow light. A photograph with blue light is reproduced in Fig. 1; the second figure is one of the ultra-violet series. In order to have sufficient absorption in the blue, a four millimeter thick cell was used with a solution containing ca 22 milligrams of protein per cc. The sedimentation constant calculated from the diagram of such a solution (Fig. 1) was $s_{20^\circ} = 10.3 \times 10^{-13}$ cm sec^{-1} dynes^{-1}. This solution is far too concentrated to give optimum results with ultra-violet illumination, but a series of photographs made with prolonged exposures showed boundaries yielding the same sedimentation constant—$s_{20^\circ} = 10.4$. It is thus apparent that the blue-absorbing molecules sediment at the same rate as those that absorb in the extreme ultra-violet; from the visual observations it is clear that the substance responsible for the absorption in the yellow comes down at substantially the same rate. These results suggest that the yellow and blue absorptions are due to the same pigment and that this pigment forms part of the protein which presumably is responsible for the ultra-violet boundary.

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[1] K. G. Stern and K. Salomon, SCIENCE, current issue.

[2] J. Biscoe, E. G. Pickels and R. W. G. Wyckoff, *Jour. Exp. Med.*, 64: 39, 1936; R. W. G. Wyckoff and J. B. Lagsdin, *Rev. Sci. Instr.*, 8: 74, 1937.

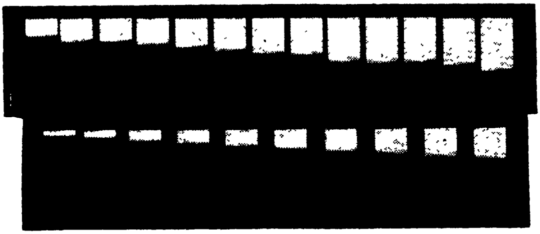

FIG. 1. Sedimentation photographs of ovoverdin using blue light. Source—high pressure mercury arc. Filter—Jena BG 12. Photographic film—Agfa Process. Centrifugal field—ca 90,000 g. Interval between photographs—5 minutes. Exposure—ca ⅓ second. Fig. 2. A series of sedimentation photographs of ovoverdin, using ultra-violet light. Source—high pressure mercury arc. Filter—chlorine plus bromine cells. Centrifugal field—ca 90,000 g. Interval between photographs—5 minutes. Exposure 1.5 seconds.

Besides this pigmented substance furnishing the boundary with $s_{20^\circ} = 10.3$, these solutions contain an appreciable amount of "unsedimentable" material transparent to visible light but showing a marked ultra-violet absorption. This impurity is not sedimented in the centrifugal fields that have been used. It must therefore be lighter than egg albumin. If it is a protein the viscosity of its solution could be great enough to retard the sedimentation of the large pigmented molecules. This possibility was tested by making sedimentation pictures of the original protein solution after dilution with two parts of water and with eight parts of physiological saline. The slightly higher values of s_{20° thus obtained—ca 10.8—are probably very near the true sedimentation constant for ovoverdin. The molecular weight of this protein can not be calculated until its diffusion constant has been determined; but the results of Svedberg[3] indicate that a protein with $s_{20^\circ} = 11$ will probably have a molecular weight of the order of 300,000.

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[3] T. Svedberg, *Chem. Rev.*, 20: 81, 1937.

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MOLECULAR SEDIMENTATION CONSTANTS OF TOBACCO MOSAIC VIRUS PROTEINS EXTRACTED FROM PLANTS AT INTERVALS AFTER INOCULATION

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PLATE 1

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In a previous paper (1) it was shown that tobacco mosaic virus proteins obtained from various sources do not all have the same molecular sedimentation rate. Such results suggested that this virus includes a family of closely related substances, the several sedimentation constants being expressions of dissimilarities in molecular shape or weight. An unexplained feature of these experiments was the observation that the rate of sedimentation seemed to depend, though somewhat irregularly, upon the age of the plants from which the protein was prepared. The present paper records a further study of the relation between sedimentation rate and length of time after plant inoculation.

Most of the samples of tobacco mosaic virus proteins used in the earlier work were purified by chemical treatments consisting of successive precipitations with ammonium sulfate at pH between 4 and 9. It has since been found that the crystalline protein can be obtained either by less drastic chemical procedures or, without chemical intervention, by precipitations in the quantity ultracentrifuge. Experience with other virus proteins isolated in these two ways has demonstrated that both the infectivity and the physicochemical properties of many are altered by prolonged chemical treatments. It thus became apparent that the same method of preparation must be used on all samples before a proper evaluation can be made of any variation of the virus protein with time after inoculation.

The writer is indebted to W. M. Stanley for the virus proteins employed in this investigation. They were portions of the same preparations whose activities and yields have been described (2). To obtain them young Turkish tobacco plants were inoculated at one time with the ordinary strain of tobacco mosaic virus. Some plants were harvested 1 week after inoculation, some after 2 weeks, and the rest at subsequent weekly intervals, the oldest being those that had grown for 13 weeks after inoculation. They were frozen when cut and were kept frozen until such time as the infectious juice was extracted by the usual procedures. From half of each batch of the freshly pressed juice the purified crystalline virus protein was prepared by two successive quantity ultracentrifugations interspersed with low speed centrifugations to remove aggregated colloidal matter (3). In the ultracentrifugations the virus protein was sedimented as a solid pellet by being held for 1 to 1½ hours in a maximum field of about 60,000 times gravity; this pellet was redissolved each time in 0.1 M phosphate buffer of pH 7. The ultracentrifuge was that recently described (4, 5), the quantity rotor being of an improved design holding from 100 to 150 cc. The virus protein, chemically prepared from each of the other half portions of juice, was crystallized by two precipitations with ammonium sulfate at pH 7 followed by one ultracentrifugation.

The sedimentation patterns of each of these two sets of virus proteins have been obtained with the analytical ultracentrifuge. The instrument (4, 6) used for these measurements was an improved model of the air-driven machine employed in the previous experiments. The original absorption method of Svedberg, which is more suited to the present purposes than the newer refractive index procedures, has been followed throughout. Duplicate series of sedimentation pictures were made and analyzed. The centrifugal field in the analytical cell was about 12,000 times gravity; ten or eleven exposures were made for each run, the interval between them being exactly 5 minutes. During a single run the speed of the centrifuge remained constant to within about 0.5 per cent. A 2 mm. thick cell was used; for this, the optimum concentration of tobacco mosaic virus protein is 1.5 mg. per cc. Sedimentation constants, referred as is customary to water at 20°, were computed from the photographs. They are collected in Table I; typical photographs are reproduced in Figs. 1 to 5.

The solutions of the first four weekly proteins prepared by quantity ultracentrifugation have the single sedimenting boundaries that indicate the presence of only one type of heavy molecule. All the proteins from plants longer infected, as well as the chemically prepared proteins of all ages beyond the 1st week, show two sedimenting boundaries and hence contain two molecular species that differ from one another in weight or shape, or both. These two components have the same sedimentation constants in all preparations in which they occur (Table I) and, judging by their ultraviolet light absorptions, are present in about the same relative amounts. The sedimentation constant of the more conspicuous component is not measurably different from that of the protein in the single boundary samples. The amount of the more rapidly sedimenting species has not yet been determined and hence it is not known whether it, too, is disease-producing; but it may be pathogenic, since no difference in infectivity was found in samples harvested after the 1st week (2).

TABLE I

Sedimentation Constants of Molecular Species in Tobacco Mosaic Virus Proteins Prepared at Various Intervals after Plant Inoculation

Time after inoculation	Sedimentation constants ($s \times 10^{-13}$)	
	Ultracentrifuged samples	Chemically extracted samples
wks.		
1	174.3	177.1
2	180.0	179.3, 203.5
3	177.6	170.6, 198.7
4	177.1	172.1, 201.9
5	173.1, 198.8	172.7, 201.1
6	172.9, 199.7	170.9, 195.4
7	175.2, 198.9	175.2, 196.6
8	175.3, 197.9	178.3, 203.2
13	174.0, 205.3	

The appearance of the heavier boundary in proteins from 2 and 3 week plants after salting-out with ammonium sulfate introduces the question of whether this component is ever present in plants or is produced in the older ultracentrifuged samples by the phosphate buffer used as solvent. In seeking for an answer it was observed that 0.1 M phosphate will cause the heavy component to appear in solutions of 2 week virus protein. Such a sample purified by ultracentrifugation was single boundaried at first, but became double boundaried after several days in the ice box. Another sample was made from plants of the same age, distilled water being used throughout as solvent. The resulting protein was of exceptional molecular homogeneity (Fig. 5) and even after standing for more than 2 months has shown no trace of the heavy component. The protein from plants infected for 13 weeks was then isolated with use of distilled water. It, too, gave a very sharp single boundary. Hence tobacco mosaic virus protein must exist in tobacco plants as a single molecular species which becomes more easily altered by salts as the plants age.

A comparison between the sedimentation results of this paper and those published earlier (1) brings out an important point. The previous preparations, as already stated, were made by chemical treatments which in most instances were more prolonged and vigorous than those now employed. Such proteins gave much more diffuse sedimenting boundaries and accordingly had far less molecular homogeneity than the proteins discussed in this paper. Their greater heterogeneity was commonly accompanied by higher mean values of the sedimentation constants; it is clear, therefore, that the different constants in the previous series were indications of alterations of the proteins during isolation rather than of their states in the plant. In order to provide a direct comparison between the present samples and one made by rigorous chemical treatment, a new photograph (Fig. 4) has been made of the sedimentation of a crystalline virus protein prepared 2 years ago. The diffuseness of boundaries in this picture compared with the boundaries of Figs. 1 and 5, together with the greater mean sedimentation constant that it gives ($s_{20°} = 223$), is an index of the molecular change brought about by such treatment.

SUMMARY

Analytical ultracentrifugal studies have been made of the sedimentation rates of virus proteins isolated from series of Turkish tobacco plants harvested 1, 2, etc., up to 13 weeks after inoculation with the ordinary strain of tobacco mosaic virus. These protein samples were prepared in two ways, half by quantity

ultracentrifugation and half by chemical methods involving ammonium sulfate precipitations, the solvent in both cases being 0.1 M phosphate buffer of pH 7. The virus proteins extracted from plants within 4 weeks of inoculation and purified by quantity ultracentrifugation consist of a single molecular species. These molecules, which are also present in all other preparations, have $s_{20^\circ} = 174 \times 10^{-13}$ cm. sec.$^{-1}$ dynes^{-1}. A second molecular component with $s_{20^\circ} = 200 \times 10^{-13}$ exists in the 2 and 3 week samples made with ammonium sulfate and develops after a few days in the corresponding ultracentrifuged proteins; it is present in the proteins from plants that have carried the disease for more than 4 weeks. The very young 1 week protein differs from those longer in the plant in that even treatment with ammonium sulfate does not produce the heavy molecular component.

Tobacco mosaic virus proteins isolated by ultracentrifugation from plants 2 and 13 weeks after inoculation are single boundaried if distilled water rather than phosphate buffer is used as solvent. The virus protein in the plants themselves hence consists of only one molecular species; it changes with length of time after inoculation, becoming more and more readily influenced by salts.

Tobacco mosaic virus proteins isolated and purified by differential ultracentrifugation or by two cautious crystallizations with ammonium sulfate show a high degree of molecular homogeneity; when water rather than phosphate is used as solvent, the ultracentrifuged proteins are unusually homogeneous. The sharply sedimenting boundaries of such proteins contrast with the diffuse boundaries and greater mean sedimentation constants given by samples subjected to more extensive treatment with chemicals.

BIBLIOGRAPHY

1. Wyckoff, R. W. G., Biscoe, J., and Stanley, W. M., *J. Biol. Chem.*, **117**, 57 (1937).
2. Stanley, W. M., *J. Biol. Chem.*, **121**, 205 (1937).
3. Stanley, W. M., and Wyckoff, R. W. G., *Science*, **85**, 181 (1937).
4. Wyckoff, R. W. G., and Lagsdin, J. B., *Rev. Scient. Instruments*, **8**, 74 (1937).
5. Wyckoff, R. W. G., *Proc. Am. Phil. Soc.*, **77**, 455 (1937).
6. Biscoe, J., Pickels, E. G., Wyckoff, R. W. G., *J. Exp. Med.*, **64**, 39 (1936).

EXPLANATION OF PLATE 1

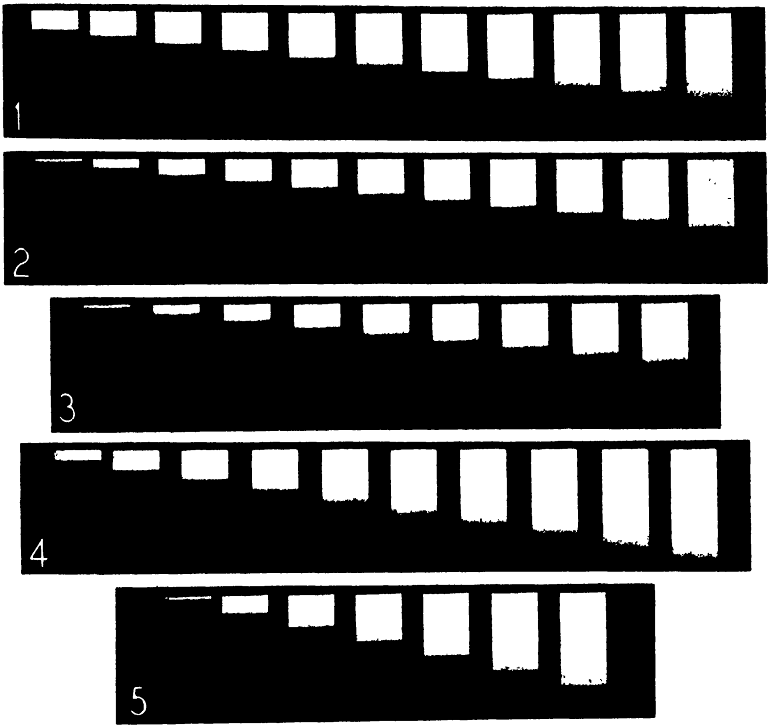
FIG. 1. The sedimentation diagram of a tobacco mosaic virus protein sample made by ultracentrifuging the juice of plants cut 1 week after inoculation. The preparation is molecularly homogeneous and shows the single boundary indicative of but one kind of heavy molecule. The 1 week chemically prepared protein and those obtained by ultracentrifugation from plants 2, 3, and 4 weeks after inoculation give sedimentation patterns indistinguishable from this. In this and Figs. 2 to 5 successive pictures were taken at 5 minute intervals. Because of the sedimentation of the absorbing virus protein molecules, more and more of the cell becomes transparent to the ultraviolet light.

FIG. 2. The sedimentation diagram of the 7 week chemically prepared sample. The two boundaries due to the two molecular components are clearly visible in the later pictures of the series. The boundary for the principal, lighter component is not quite so sharp as in Fig. 1.

FIG. 3. The sedimentation diagram of the 13 week ultracentrifuged protein. This pattern is double boundaried, though the second, heavier one is harder to see than in Fig. 2.

FIG. 4. The sedimentation diagram of a solution of a crystalline virus protein made by more extensive chemical treatments. The very diffuse boundary is indicative of a considerable molecular heterogeneity; the greater rate at which it moves outward in the cell corresponds to the greater observed sedimentation constant. The presence of much colloidal matter in this sample is shown by the gradual increase in transparency of the cell below the boundary.

FIG. 5. The sedimentation diagram of a 2 week protein made by ultracentrifugation with water instead of phosphate buffer as solvent. The single boundary remains perfectly sharp even in the final picture. The greater apparent rate of sedimentation in this diagram is due to the use of a higher centrifugal field.



(Wyckoff: Sedimentation of mosaic virus)

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PROPERTIES OF THE TYPE SPECIFIC PROTEINS OF ANTIPNEUMOCOCCUS SERA

I. THE MOUSE PROTECTIVE VALUE OF TYPE I SERA WITH REFERENCE TO THE PRECIPITIN CONTENT

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Other factors being equal it might be expected that any specific immunological value of a given immune serum should be proportional to the amount of the specific antibody. For example it might be held that the effectiveness of a given antipneumococcus serum in protecting animals against pneumococcus infection could be judged by estimating the amount of protein specifically precipitable with the homologous capsular polysaccharide, that is, the amount of antibody protein (precipitin). This thesis is presumably tenable only if the injection of various animals with a single antigen gives rise to a single antibody, or to a mixture in fixed proportions of antibodies dominantly specific but differing in one or more chemical or immunological properties. Present evidence indicates that agglutination, precipitation, and complement fixation are due to the same substances in the serum. It has not as yet been demonstrated, however, that in a single serum there exists only one antibody substance specific for a given antigen.

This question is important from the practical as well as from the theoretical point of view. The clinician has generally held to the significance of tests for potency in which animals are used. This view has developed as a result of the fact that antipneumococcus sera of certain types afford excellent protection to the experimental animal and at the same time are effective therapeutic agents in lobar pneumonia. With other types of pneumococci, notably Type III, the immune sera may possess considerable antibody potency as judged by agglutination titer and yet be lacking in ability to protect animals against large numbers of pneumococci. Sera of these types have also proven less successful in the clinic.

On the other hand, it has long been recognized that evaluation by animal methods is difficult and simpler means of potency estimations have been sought. In spite of many excellent studies on this subject (1–6), it is as yet by no means certain that an absolute parallelism exists between *in vitro* and *in vivo* results. It is generally held that this uncertainty is related to the lack of precise methods for determining protective potency.

The present paper consists of an experimental appraisal of the mouse protection test, its quantitative possibilities, and of the relation between the amount of antibody quantitatively determined by *in vitro* methods and the amount of demonstrable protective antibody.

EXPERIMENTAL

These experiments deal only with Type I antipneumococcus sera.

Sera.—Type I antipneumococcus horse sera were obtained through the courtesy of Dr. Augustus B. Wadsworth of the New York State Department of Health and Dr. W. E. Bunney of the Michigan State Department of Health. The standard serum used is that designated as 425 and has been described by Kirkbride, Hendry, and Murdick (7). According to titrations carried out by these workers this serum contains 1000 mouse protective units per cc. by comparison with the National Institute of Health standard serum. Type I antipneumococcus rabbit sera were produced in this laboratory.

Capsular Polysaccharide.—Viscous Type I capsular polysaccharide was prepared by the method of Heidelberger, Kendall, and Scherp (8). Analyses of this preparation showed nitrogen 5.58 per cent; acetyl 7.35 per cent; and ash calculated as sodium 3.98 per cent. In 0.1 per cent solution in 0.85 per cent NaCl the polysaccharide had a relative viscosity of 1.22.[1] For the preparation of stock solutions the polysaccharide was dried to constant weight at low temperature and dissolved in saline (containing merthiolate 1–10,000). This solution was then diluted to give a final polysaccharide concentration of 0.1 per cent.

Determination of Specifically Precipitable Nitrogen.—Specific precipitates were prepared from the various sera studied by the addition to them of varying amounts of the capsular polysaccharide. The method of Heidelberger and Kendall (9) was followed and all precipitates were prepared in the cold and washed twice with ice-cold saline. In every instance increasing amounts of polysaccharide were added to constant amounts of serum and the total volume of each reacting mixture was kept constant by the addition of cold saline. The specific precipitates were digested and their nitrogen content determined by the gasometric micro method of Van Slyke (10) as modified by Kirk, Page, and Van Slyke (11). The precise details of the application of this method have been previously described (12). Duplicate precipitates were prepared and consequently the figures given for precipitated nitrogen in the various tables are the mean of two determinations unless otherwise indicated. Aliquots of the initial supernatant solutions decanted from the precipitates after the first centrifugation were tested separately for the presence of uncombined polysaccharide and uncombined antibody. In each instance the same serum was used for these tests as had been used in the formation of the initial precipitates. In this way it was possible to determine the position of the equivalence zone for each serum.

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[1] These chemical studies were kindly carried out by Dr. Walther Goebel.

Inasmuch as the precipitates were prepared from constant amounts of serum in each instance and since the volume of the reacting mixtures was kept constant, there was no appreciable variation in the concentration of lipids in the individual preparations. From the results of previous work it is therefore assumed that the amount of lipid adsorbed by each precipitate within any single experiment was constant. Consequently it has not been necessary to make any correction for the very small amount of lipid nitrogen in the precipitates since this would be expected to be constant for any particular serum.

Culture.—Pneumococcus Type I, original Neufeld strain. The culture is maintained in rabbit blood broth at 37°C. with transfers every 2nd day. Under these conditions the virulence of the culture remains practically constant, that is, there is an almost constant number of virulent organisms in a given volume of a culture of a given age. The virulence is such that 0.000,000,01 cc. of culture given intraperitoneally, produces a fatal infection in mice. This amount of culture usually represents 3 microorganisms.

Mice.—Throughout this work the so called Rockefeller strain of white mice has been used. This stock is highly inbred and the host factors involved are fairly well understood. All protection tests have been carried out with mice weighing from 17 to 23 gm.

Mouse Protection Test.—Tests for the protective potencies of the various sera were carried out by the method described by Kirkbride, Hendry, and Murdick (7) and the results analyzed by the method of Muench (cited by Lloyd, Theiler, and Ricci (13)).

This protection method differs from certain others in general use by the fact that a relatively large amount of culture is used as infective inoculum. This amount of culture is so chosen as to avoid the *Schwellenwert* and yet be in the range in which the titer limit follows roughly the law of multiple proportions (14). In order to determine the value of the unknown serum a simultaneous titration is carried out with a serum of known potency which serves as a standard for comparison. In practice the following technical procedure has been observed.

1. The culture is diluted in broth so that 0.5 cc. of the dilution contains 0.1 cc. of the original 18 hour blood broth culture. (This amount of culture contains approximately 30,000,000 pneumococci and in this instance represents a corresponding number of minimum lethal doses). The usual virulence controls are carried out.

2. At least three different dilutions of each serum are used in each test. The sera are progressively diluted × 2, the precise dilutions being determined by exploratory tests.
3. For the actual test at least 5 mice are injected with each combination of serum and culture. Each mouse receives 0.5 cc. culture dilution and 0.5 cc. of the appropriate serum dilution, these being mixed in the syringe and injected into the peritoneal cavity.
4. Mice surviving for 96 hours are considered as protected by the corresponding amount of serum.

Estimation of Protective Potencies

The most difficult feature of any method involving biological variables is the accurate evaluation of the results obtained. Various workers have adopted various end-points. Thus the minimum amount of serum which will protect 60 per cent of mice can be taken as the end-point. There are three objections to this and similar criteria. First, this method gives end-points which are as widely spaced as are the dilutions of serum, that is, only rough approximations of possible numerical values can be obtained. Secondly, the result is actually dependent on very few mice, those in the group receiving the particular protecting dilution and those of the group receiving the next lower amount of serum. Thirdly, results are not always sufficiently regular to permit an adequate determination of the end-point. Thus, with 5 or even 10 mice per dilution, two amounts of serum, one being twice the other, may result in the same percentage survival. Several attempts have been made to overcome this end-point difficulty. It seemed very desirable to obtain a sensitive numerical end-point based not on the small group of mice at any particular dilution level but rather one which would take into account the fate of all mice irrespective of the amount of serum.

Since the usual data, if plotted, give curves with much sharper slopes in the region of 50 per cent survival, it must be generally agreed that in this type of biological work this is the only logical end-point. After several different methods had been tried the so called Muench 50 per cent end-point accumulation method was adopted.

This method was devised by Dr. Hugo Muench of The Rockefeller Foundation and is extremely simple in application. It has been used in the analysis of the results of protection tests in yellow fever work by Lloyd, Theiler, and Ricci (13) and in studies on the titration of vaccine virus by Parker and Rivers (15). The latter authors discuss the validity of the method and the system used for obtaining the numerical end-point. In brief, the method is as follows: The survivals and deaths for each serum dilution are separated. The figures in each column are then accumulated, each column being added beginning at the smaller end. If the resulting figures are then plotted against serum dilutions the lines will cross at a point which represents the dilution of serum which should be used to bring about a survival rate of 50 per cent. Actually it is unnecessary to plot these data since a simple calculation will provide the figure.

An illustration of the actual application of this method is provided in Table I. It will be noted that in this titration 10 mice have been used for each dilution of serum. The results in terms of percentage survival for each dilution are shown in column 4. When these figures are directly plotted a line connecting them forms a smooth curve and the 50 per cent end-point is easily determined. Results of this order are, however, very exceptional. In the fifth and sixth columns the survivals and deaths have been accumulated as described above. The percentage survival of each serum level has been calculated and is shown in column 7. On inspection it is obvious that the desired 50 per cent end-point lies between the serum dilutions 1–40 and 1–80. The exact point is obviously 23/46 or 0.50 of the distance between 1–40 and 1–80. Since the progression in serum dilutions is geometric it is necessary either to convert the basic dilution number (40 in this instance) into a log factor for multiplication, or much simpler in operation, to convert the factor 0.50 into a proportional factor by reference to a progression chart. The calculated end-point in this example is 1–56.

TABLE I

Specimen Mouse Protection Test with Determination of 50 Per Cent End-Point by the Muench Accumulation Method

Serum dilution	Result			Accumulation			Calculated 50 per cent end-point
	Survived	Died	Survival	Survivals	Deaths	Survivals	
			per cent			*per cent*	
1–10	10	0	100	30	0	100	
1–20	9	1	90	20	1	95	
1–40	7	3	70	11	4	73	
1–80	3	7	30	4	11	27	1–56
1–160	1	9	10	1	20	5	

For the actual estimation of the potency of a given serum it is necessary to carry out protection titrations of the unknown serum and of the standard serum at the same time. This requirement tends to overcome the disadvantages of slight variations in the number of microorganisms in a culture from day to day. The end-point for each serum is then determined and by proportional calculation the potency of the unknown serum can be estimated. An example of actual titrations on the same serum at different times is given in Table II. Five independent titrations of an unknown serum have

TABLE II

Comparative Titrations for the Estimation of the Mouse Protective Potency of Lot AA Type I Antipneumococcus Horse Serum

Date	Standard Type I antipneumococcus horse serum (No. 425) 1000 units per cc.								Type I antipneumococcus horse serum lot AA								Estimated potency of serum AA units per cc.
	Dilutions	Results			Accumulation			Calculated 50 per cent end-point	Dilutions	Results			Accumulation			Calculated 50 per cent end-point	
		Sur-vived	Died	Sur-vival rate	Sur-vivors	Deaths	Sur-vivals			Sur-vived	Died	Sur-vival rate	Sur-vivors	Deaths	Sur-vivals		
				per cent			*per cent*					*per cent*			*per cent*		
Jan. 23	1–20	5	0	*100*	11	0	*100*	1–64	1–30	2	2	*50*	8	2	*80*	1–87	1360
	1–40	4	1	*80*	6	1	*86*		1–60	4	0	*100*	6	2	*75*		
	1–80	2	3	*40*	2	4	*33*		1–120	1	3	*25*	2	5	*29*		
	1–160	0	5	*0*	0	9	*0*		1–240	1	3	*25*	1	8	*11*		
Mar. 13	1–40	13	2	*87*	20	2	*91*	1–70	1–32	10	0	*100*	22	0	*100*	1–109	1560
	1–80	7	8	*47*	7	10	*41*		1–64	6	4	*60*	12	4	*75*		
	1–160	0	10	*0*	0	20	*0*		1–128	6	4	*60*	6	8	*43*		
									1–256	0	5	*0*	0	13	*0*		
Apr. 14	1–40	4	1	*80*	8	1	*89*	1–94	1–40	5	0	*100*	11	0	*100*	1–137	1460
	1–80	4	1	*80*	4	2	*67*		1–80	3	2	*60*	6	2	*75*		
	1–160	0	5	*0*	0	7	*0*		1–160	3	2	*60*	3	4	*43*		
Apr. 27	1–40	5	0	*100*	7	0	*100*	1–71	1–40	4	1	*80*	8	1	*89*	1–90	1270
	1–80	2	3	*40*	2	3	*40*		1–80	3	2	*60*	4	3	*57*		
	1–160	0.	5	*0*	0	8	*0*		1–160	1	4	*20*	1	7	*13*		
May 6	1–40	3	2	*60*	3	2	*60*	1–45	1–40	2	3	*40*	6	3	*67*	1–62	1380
	1–80	0	5	*0*	0	7	*0*		1–80	2	3	*40*	4	6	*40*		
	1–160	0	5	*0*	0	12	*0*		1–160	2	3	*40*	2	9	*18*		
Mean number of mouse protective units per cc. of serum AA......																	1405

been carried out simultaneously with titrations of the known or standard serum.

The data presented in Table II are fairly representative of the order of results generally obtained. Some of these titrations would present an impossible problem were it necessary to judge the end-points on a simple survival basis. Furthermore the results show the great advantages of simultaneous titrations of unknown and standard for although the actual end-points for each serum vary widely the variations are generally in the same direction for both sera at the same time. Thus when the actual unitage value is calculated one finds a rather close agreement considering the number of mice used in each titration. It is believed that the mean value, 1405 protective units per cc., is as representative of the actual protective potency of the serum as any figure obtainable by any biological method. The probable error involved in titrations such as that illustrated with five separate determinations is about 10 per cent although no accurate estimate of this factor can be made without much longer series of titrations.

TABLE III

Mouse Protective Potencies and Specifically Precipitable Antibody of Nine Type I Antipneumococcus Horse Sera

Group	Serum lot	Character	Protective units per cc.	Maximum specifically precipitable nitrogen	Protective units per mg. of specifically precipitable nitrogen	Mean protective ratio for each group
				mg. per cc.		
A	58	Raw, single	440	0.765	575	540
	57	Raw, single	550	1.018	540	
	50	Raw, pooled	760	1.510	505	
	14	Concentrated	3145	5.798	545	
B	49	Raw, pooled	500	0.645	775	800
	59	Raw, single	870	1.081	805	
	60	Raw, single	1000	1.229	815	
	AA	Raw, pooled	1405	1.844	760	
	13	Concentrated	6100	7.125	855	

Antibody Content of Type I Antipneumococcus Horse Sera

With a measure of validity having been shown for the method of estimation of protective potencies and for the analysis of the results it is now possible to proceed to a comparison of the protective potency and the amount of specific antibody (precipitin) demonstrable *in vitro.*

These two forms of experimental analysis have been carried out on nine Type I antipneumococcus horse sera and the results are shown in Table III.

For the estimation of mouse protective potencies several titrations were carried out with each serum, the average number in this series being slightly over 4. The average number of mice per serum, exclusive of those involved in the simultaneous titration of the standard serum, was 73. These repeated titrations were necessary in order to secure numerical end-points with some degree of precision. Similarly the estimations of maximum specifically precipitable nitrogen represent data secured from complete precipitin curves, each point on which represents an average of determinations on two duplicate precipitates.

The nine antipneumococcus horse sera included in Table III fall into two groups dependent upon the number of mouse protective units per mg. of specifically precipitable nitrogen (hereinafter termed the *protective ratio*). Thus group A includes four sera, both raw and concentrated, with potencies ranging from 440 to 3145 units per cc. of serum. In the number of units per mg. of specifically precipitable nitrogen (protective ratios) however, these sera differ but slightly, the range being from 505 to 575 with a mean of 540 units per mg.

In contrast are the results with the sera of group B. Although the mouse protective potencies of these sera range from 500 to 6100 units per cc., the protective ratios are rather uniform ranging from 760 to 855 units per mg. with a mean value of 800.

It is perhaps merely fortuitous that the mean protective ratios of these two groups of Type I antipneumococcus horse sera stand in the approximate relation of 2:3. It does not seem likely however that the actual protective ratios of the various sera are matters of chance. The difference between the results of the two groups is considerably greater than the outside probability of error in either protection or nitrogen estimations.

Several possible sources of error do, however, exist. The validity of the mouse protection titrations has already been discussed, and while some allowance must be made for the probability of error in each instance, this possible deviation is not sufficiently great to account for the differences between the two groups. A second possibility is that the amounts of specifically precipitable nitrogen do not accurately represent the amounts of specific antibody. These determinations were, however, very carefully carried out and the results of extensive checking show that with any given lot of pneumococcus polysaccharide the maximum amount of specifically precipitable nitrogen as determined by the standard procedure is a fixed characteristic of a given serum. Whether one is warranted in assuming that this precipitable nitrogen represents only specific antibody is another matter. It has been shown by Heidelberger and Kendall (16), for example, that if Type II capsular polysaccharide is added to a serum containing both Type I and Type II antibodies, both will be found in the resulting precipitates. An example of a quantitative experiment designed to determine the extent of this non-specific precipitation is shown in Table IV. Type I capsular polysaccharide was added to Type I and Type II sera separately and after these had been mixed. It will be noted that while the addition of Type I capsular polysaccharide to the Type II serum gave no precipitate the addition of the carbohydrate to the mixture of the two sera gave a nitrogen result higher by 7.2 per cent than was obtained by the addition of the polysaccharide to the Type I serum. This is regarded as a significant increase but the amount is not great enough even under the conditions of this experiment to affect seriously the general results shown in Table III.

TABLE IV

The Influence of Heterologous Antibodies on the Apparent Amount of Specifically Precipitable Nitrogen

Type I antipneumococcus horse serum	Type II antipneumococcus horse serum	Type I capsular polysaccharide	Precipitable nitrogen
cc.	*cc.*	*mg.*	*mg.*
1.0	—	0.25	1.490
1.0	1.0	0.25	1.597
—	1.0	0.25	0.000

Precipitation carried out at 4°C. according to the method of Heidelberger and Kendall (9).

Antibodies other than the type specific anticarbohydrate are present in practically every Type I antipneumococcus horse serum. Some of these, such as those directed against the pneumococcus protein and the somatic carbohydrate (C substance) are present in amounts so small as to affect the results only to a very slight extent even were a considerable proportion included in the immune precipitate. Tests as to the monovalence of the immune horse sera were carried out and it was found that only one, No. 58, gave any reaction with the capsular polysaccharides of other pneumococcus types; this serum had antibodies which reacted with the capsular polysaccharide of Type II Pneumococcus, although the reaction was faint. It would therefore appear that the quantitative results have not been seriously affected by non-specific reactions.

Since there appears to be no obvious experimental error sufficient to account for the observed differences between the two groups of immune horse sera another explanation must be sought. In the absence of definitive evidence any explanation must, however, be of a hypothetical nature.

As a first possibility it may be that certain of these sera contain substances other than the specific antibody which largely condition the *in vivo* efficacy of these antibodies. From the results of studies on the relation of lipids to immunological reactions, one might hold that an abnormal lipid pattern would tend to inhibit the protective action of the serum (17). This possibility seems somewhat unlikely, however, in view of the fact that each group included both pooled sera and sera from single bleedings. Furthermore if some inhibitor were present in the sera of group A it would seem unlikely that the amount would be so constant with respect to the amount of precipitable antibody as to give the consistent results obtained. If the inhibitor were intimately and quantitatively associated with the antibody molecules this hypothesis might be tenable.

TABLE V

Mouse Protective Potencies and Specifically Precipitable Antibody of Seven Type I Antipneumococcus Rabbit Sera

Serum lot	Protective units per cc.	Maximum specifically precipitable nitrogen	Protective units per mg. of specifically precipitable nitrogen	Mean protective ratio
		mg. per cc.		
22	1070	0.938	1140	1170
28	1130	0.986	1145	
27	1190	1.108	1075	
A7	1270	1.128	1125	
26	1390	1.116	1245	
25	1830	1.533	1195	
24	1910	1.510	1265	

All lots represent pooled bleedings and the sera have not been concentrated.

A second explanation is that certain horses may produce antibodies having greater protective values. Here again the matter of sera from single and pooled bleedings would seem to make this possibility slight.

A third possibility is that the antibodies in a given immune horse serum, although reacting with the specific polysaccharide, may in fact represent a series of substances possessing different avidities, different degrees of specificity, different protective values, and perhaps even different chemical properties. It is possible that an immune serum may contain a mixture of specifically reactive antibodies varying in one or more of these properties.

Antibody Content of Antipneumococcus Rabbit Sera

Determinations of protective potencies and of maximum amounts of specifically precipitable nitrogen have been carried out with seven Type I antipneumococcus rabbit sera. The results of these determinations are presented in Table V.

The estimations of protective potencies are based on an average of 4.7 titrations per serum, with an average number of 77 mice per serum, exclusive of those involved in the simultaneous titration of the standard serum.

It will be noted that the protective values of the immune rabbit sera range from 1070 to 1910 units per cc. but that the number of protective units per mg. of specifically precipitable nitrogen is relatively constant, averaging 1170. Thus Type I antipneumococcus rabbit sera appear to be uniform in their protective ratios. This result stands in contrast to those obtained with antipneumococcus horse sera.

There is reason therefore to believe that in the case of immune rabbit serum the protective potency can be directly estimated from determinations of the maximum amount of specifically precipitable protein. This, however, is true only under certain definite conditions. It has been clearly pointed out by Heidelberger, Kendall, and Scherp (8) that various preparations of capsular polysaccharide vary in their capacity to precipitate the antibodies of antipneumococcus rabbit serum. This variation is so great that it is necessary to test each polysaccharide preparation. It is believed, however, that if a given polysaccharide preparation is standardized against one serum and if this serum has been fully appraised in a mouse protection test, one should be able to standardize all other immune rabbit sera of the same type on the basis of the known precipitating properties of the given lot of polysaccharide. It is known, for example, that the lot of polysaccharide used in these experiments will precipitate approximately 92 per cent of the specific protective antibody from a given antipneumococcus rabbit serum.

The general differences between the protective ratios of immune horse and immune rabbit sera can perhaps be partially explained. With Bauer (18) it has been shown by ultrafiltration that the horse antibodies are in general larger than those of immune rabbit serum. Heidelberger and Pedersen (19) have demonstrated similar differences by the use of the ultracentrifuge. Although in certain details the results by the two methods are not in complete accord, there is now little doubt but that a difference in size does exist. If the rabbit antibody is actually smaller than that of the horse there should be in a given mass of rabbit antibody a much greater reactive surface and therefore one would expect that a greater amount of carbohydrate could be bound in proportion to the amount of antibody. At the present time it is not possible to conclude that this explanation accounts for the observed differences in protective ratios, but the facts are suggestive.

SUMMARY

The ability to carry out with some measure of precision mouse protection tests for the estimation of potency of antipneumococcus sera has made possible the correlation of the protective potency with the amount of specifically precipitable protein. With antipneumococcus rabbit sera these protective ratios are relatively constant and higher than those with immune horse serum. Type I antipneumococcus horse sera, on the other hand, show no such constancy but fall into two groups; and there is as yet no simple method for determining to which group a serum belongs.

BIBLIOGRAPHY

1. Heidelberger, M., Sia, R. H. P., and Kendall, F. E., *J. Exp. Med.*, 1930, **52**, 477.
2. Felton, L. D., *J. Immunol.*, 1931, **21**, 34.
3. Smith, W., *J. Path. and Bact.*, 1932, **35**, 509.
4. Cotoni, L., and Pochon, J., *Ann. Inst. Pasteur*, 1936, **57**, 221.
5. Barnes, L. A., Clarke, C. M., and Wright, E. C., *J. Immunol.*, 1936, **30**, 127.
6. Felton, L. D., and Stahl, H. J., *Bull. Nat. Inst. Health, U. S. P. H. S., No. 169*, 1937.
7. Kirkbride, M. B., Hendry, J. L., and Murdick, P. P., *Am. J. Hyg.*, 1936, **23**, 187.
8. Heidelberger, M., Kendall, F. E., and Scherp, H. W., *J. Exp. Med.*, 1936, **64**, 559.
9. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **61**, 559.
10. Van Slyke, D. D., *J. Biol. Chem.*, 1927, **71**, 235.
11. Kirk, E., Page, I. H., and Van Slyke, D. D., *J. Biol. Chem.*, 1934, **106**, 203.
12. Horsfall, F. L., Jr., and Goodner, K., *J. Exp. Med.*, 1936, **64**, 583.
13. Lloyd, W., Theiler, M., and Ricci, N. I., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1936, **29**, 481.
14. Goodner, K., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1935, **62**, 359.
15. Parker, R. F., and Rivers, T. M., *J. Exp. Med.*, 1936, **64**, 439.
16. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1936, **64**, 161.
17. Goodner, K., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1936, **64**, 377.
18. Goodner, K., Horsfall, F. L., Jr., and Bauer, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 617.
19. Heidelberger, M., and Pedersen, K. O., *J. Exp. Med.*, 1937, **65**, 393.

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PROPERTIES OF THE TYPE SPECIFIC PROTEINS OF ANTIPNEUMOCOCCUS SERA

II. IMMUNOLOGICAL FRACTIONATION OF TYPE I ANTIPNEUMOCOCCUS HORSE AND RABBIT SERA

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In the preceding paper of this series (1) it was reported that the amount of specific antibody precipitable by the capsular polysaccharide is related to the mouse protective value of Type I antipneumococcus serum only under certain well defined conditions. Thus with Type I antipneumococcus rabbit serum there was a direct proportionality between the two determined values. The protective ratio for immune rabbit serum, that is, the number of protective units per mg. of specifically precipitable nitrogen, was, however, higher than that for Type I antipneumococcus horse serum. Moreover, it was found that with the latter there existed at least two different protective ratios. Thus antipneumococcus rabbit sera average 1170 mouse protective units per mg. of specifically precipitable nitrogen, whereas horse sera fall into two groups having average values of 540 and 800 units per mg., respectively.

Several hypotheses were advanced as possible explanations for the differences in the protective ratios of different lots of antipneumococcus horse sera. It was reasoned that if, according to the generally accepted thesis, the horse had formed only one antibody specific for the pneumococcus capsular polysaccharide, the protective ratios of the sera might differ because of certain secondary factors. It is known, for example, that the protective action of antipneumococcus horse serum can be readily modified by admixture with certain lipids in very small amounts (2). The consistency of the results within the groups would necessitate a degree of uniformity in distribution of secondary substances which would seem to render this possibility somewhat unlikely.

It was also suggested as a possibility that more than one substance in the immune horse serum might possess the capacity of reacting with the capsular polysaccharide but that each of these possible antibodies might possess characteristically different capacities to confer passive protection on mice. It was held that if this were correct immune horse sera might represent equilibrated mixtures of antibodies, dominantly type specific, but varying in degree as to protective action. These various hypotheses are somewhat difficult of experimental proof but two forms of experimentation have been devised to determine if all type specific antibodies in a given antipneumococcus serum possess the same capacity to confer protection upon mice. The first approach is based on the fractional precipitation of the antibody from serum by the addition of various amounts of specific polysaccharide. From analyses of the amount of protein specifically precipitated and of the amount of protective antibody remaining in the absorbed serum, it is possible to calculate the protective value of each fraction of antibody precipitated. The data derived from experiments of this order form the subject matter of the present paper.

A second and more direct approach has also been employed, namely, the attempt to isolate antibody proteins in more or less pure form with analyses of the protective values of the variously isolated fractions. The results of this study will be presented in a subsequent paper.

EXPERIMENTAL

Materials and Methods.—A detailed description of the immune sera, of the capsular polysaccharide preparation, and of the general methods of procedure used in the present study has been given in the preceding paper (1). In brief, the method for the determination of the amount of specifically precipitable nitrogen in the immune precipitates is that proposed by Heidelberger and Kendall (3). Precipitates are prepared in the cold and after 18 hours at 4°C. are carefully washed twice with ice-cold saline. Protein precipitated is determined as nitrogen. For mouse protection tests the method of Kirkbride, Hendry, and Murdick (4) has been used and the results analyzed by the method of Muench (5). Experimental evidence supporting the accuracy of the technical procedures employed has been given in the preceding paper (1).

Immunological Fractionation of Antipneumococcus Horse Serum

Heidelberger and Kendall in a series of papers have demonstrated the quantitative interrelationships between the amount of capsular polysaccharide added to a specific immune serum and the resultant amount of immune precipitate (6). It is possible to remove a definite and predictable quantity of the specific antibody by the addition of a known amount of polysaccharide, and to estimate the amount of antibody remaining in solution either by difference or by direct determination. At the same time it is possible to estimate the amount of protective antibody remaining in the absorbed serum by the tests and analytical procedures previously described (1). If a series of partial absorptions is carried out and the supernatant fluids so remaining after each precipitation are analyzed, it is possible to calculate by difference the protective potency of the antibody removed by each addition of specific polysaccharide.

In the first experiment Type I antipneumococcus horse serum, lot AA was used. By repeated mouse protection titration against a standard serum the test serum has been found to contain approximately 1405 protective units per cc.

To each of a large series of tubes was added exactly 2.0 cc. of a 1:2 dilution of sediment-free serum and the tubes were then chilled. Various amounts of chilled capsular polysaccharide solution were then added as indicated in Table I. The volumes were then made up precisely to 4.0 cc. with cold saline. After 18 hours at 4°C. all tubes were centrifuged for 1 hour in the cold. The supernatant fluids were then decanted and those from duplicate tubes were pooled. The precipitates were carefully washed twice with ice-cold saline and the precipitated protein determined as nitrogen. The analyses, carried out in quadruplicate, therefore represent the amount of nitrogen specifically precipitable from 1 cc. of serum by the indicated amount of polysaccharide.

Simultaneous mouse protection tests were next carried out with the supernatant solutions which represented exactly a 1-4 dilution of the original serum. The number of mouse protective units in the supernatant fluids was calculated by reference to the unitage potency of the unadsorbed serum as previously determined by simultaneous titrations against a standard.

Table I shows the amounts of nitrogen specifically precipitated by the various amounts of capsular polysaccharide both as mg. per cc. of antiserum and in per cent with reference to the maximum amount of specifically precipitable nitrogen. From these data have been calculated the amounts of specific antibody nitrogen which in theory should still be present in the supernatant fluids. That all precipitates (except VII) were formed on the antibody excess side of the equivalence zone was shown by the fact that none of the other supernatant fluids contained immunologically demonstrable capsular polysaccharide.

The results of the mouse protection tests on the supernatant solutions in terms of dilution end-points (Muench 50 per cent system) are also shown. From these figures by proportional calculation have been derived the number of mouse protective units remaining in the supernatant solutions. The last column gives the number of protective units per mg. of specifically precipitable nitrogen.

TABLE I

Fractional Precipitation of Antipneumococcus Horse Serum with Specific Capsular Polysaccharide and the Determination of the Protective Potency of the Unprecipitated Antibody in the Supernatant Fluid

Supernatant fluids		N_2 specifically precipitated		Supernatant fluids				
				Specific N_2 (by difference)		Mouse protection		
Designation	Obtained after addition of capsular polysaccharide mg. per cc. of serum	Mg. per cc.	Per cent of maximum specifically precipitable nitrogen	Mg. per cc.	Per cent of maximum specifically precipitable nitrogen	Dilution end-point	Calculated units per cc. referred to original serum	Protective units per mg. of specific N_2 (protective ratio)
I	0	0	0	1.844	100.0	1–109.4	1405	760
II	0.025	0.559	30.3	1.285	69.7	1–87.7	1125	876
III	0.05	0.923	50.1	0.921	49.9	1–64.0	822	893
IV	0.06	1.124	61.0	0.720	39.0	1–39.7	510	708
V	0.08	1.342	72.8	0.502	27.2	1–13.6	175	349
VI	0.10	1.571	85.3	0.273	14.7	1–4.0	51	187
VII	0.25	1.844	100.0	0	0	—	0	—

From these results it will first be noted that the admixture of 0.25 mg. capsular polysaccharide with 1.0 cc. of serum removed all of the protective antibody. Each addition of polysaccharide removed a certain amount of protective antibody, but the relationship between protective potency and specifically precipitable protein remaining in solution is not linear. Thus it will be noted that with the removal of the first 30 per cent of specifically precipitable nitrogen the antibody remaining in solution showed a higher protective ratio than that in the original unabsorbed serum. The direction of this change, however, was reversed following the removal of 60 per cent of the precipitable antibody, and as more and more antibody was removed the protective ratio of that remaining in solution became progressively lower, until finally when 84 per cent had been precipitated the antibody remaining possesses a potency of only 187 protective units per mg.

It is obvious that all supernatant fluids previous to VI contained antibody of low protective potency. It is possible, however, to arrive at the actual protective potency of each fraction of precipitable antibody by a process of subtraction. The results of these calculations are shown in Table II and presented diagrammatically in Text-fig. 1.

TABLE II

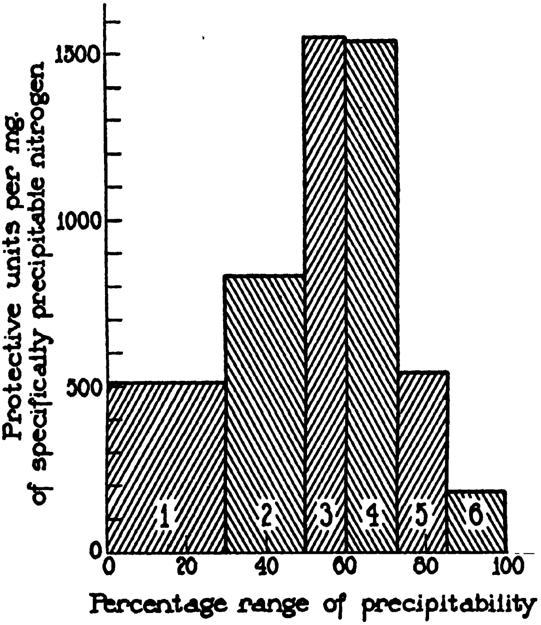
Analyses of Protective Potencies of Various Fractions of the Specifically Precipitable Antibody of Type I Antipneumococcus Horse Serum

Fraction		Position in precipitable range	Specific nitrogen content of fraction (by difference from Table I)	Protective units in fraction (by difference from Table I)	Protective ratios: Units per mg. specific N_2
Designation	Reference supernatant designation				
		per cent			
1	I-II	0 -30.3	0.559	280	505
2	II-III	30.3-50.1	0.364	303	830
3	III-IV	50.1-61.0	0.201	312	1550
4	IV-V	61.0-72.8	0.218	335	1540
5	V-VI	72.8-85.3	0.229	124	540
6	VI-VII	85.3-100.0	0.273	51	187

It will be noted that the antibody precipitated by the addition of the smallest amount of capsular polysaccharide possessed a protective ratio (505 units per mg.) much lower than that of the antibody in the unabsorbed serum (760 units per mg.). Similarly the last fraction precipitated (No. 6) possessed an extremely low protective potency (187 units per mg.). Between these two low protective ratios, however, there exists a range in which the mouse protective value is very high, approximately 1550 units per mg. of specifically precipitable nitrogen which is almost exactly twice the figure for the unabsorbed serum.

Repeated experiments as well as estimations of the probable errors in the work lead to the conclusion that although the actual figures may not be absolute the comparative values are correct.

It is quite apparent from these results that those substances in antipneumococcus horse serum which are considered as specific antibodies do not possess equal properties as regards their capacity to protect mice against infection.



TEXT-FIG. 1. Protective potencies of various fractions of the specifically precipitable antibody of Type I antipneumococcus horse serum.

These results might be interpreted as demonstrating the existence in an immune horse serum of a large series of antibodies of varying potencies. A simpler conception would be that in antipneumococcus horse serum there exist at least three substances possessing all of the properties of specific antibodies. That which is first precipitated upon the addition of polysaccharide might be thought of as possessing a great avidity[1] but for some reason a low protective value. That which occupies the intermediate position has less avidity but a very high mouse protective value. That which is last precipitated has the lowest avidity and also the lowest protective value. The figures obtained in this analysis undoubtedly do not represent the actual values inasmuch as these fractions probably still represent mixtures.

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[1] The term avidity is here used in the more commonly accepted sense of rate of visible reaction rather than with reference to antigen-antibody union proper.

Immunological Fractionation of Antipneumococcus Rabbit Serum

A similar procedure of immunological fractionation has been carried out with Type I antipneumococcus rabbit serum and the results are presented in Table III.

TABLE III

Fractional Precipitation of Antipneumococcus Rabbit Serum with Specific Capsular Polysaccharide and the Determination of the Protective Potency of the Unprecipitated Antibody in the Supernatant Fluid

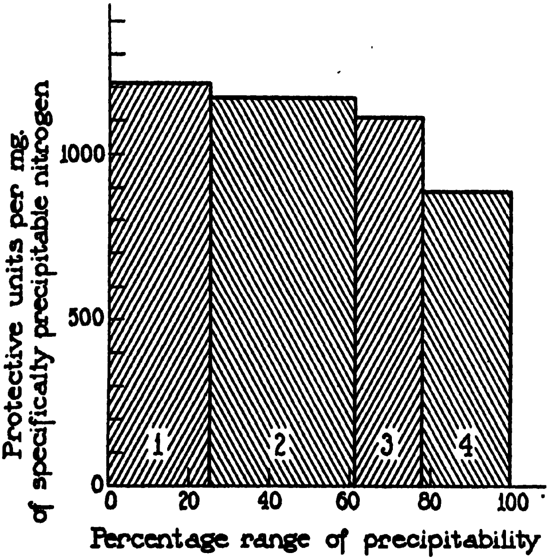
Supernatant fluids		N_2 specifically precipitated		Supernatant fluids					
Designation	Obtained after addition of capsular polysaccharide mg. per cc. of serum	Mg. per cc.	Per cent of maximum specifically precipitable nitrogen	Specific N_2 (by difference)		Mouse protection		Protective units per mg. of specific N_2 (protective ratio)
				Mg. per cc.	Per cent of maximum specifically precipitable nitrogen	Dilution end-point	Calculated units per cc. referred to original serum	
I	0	0	0	1.533	100.0	1–106.8	1830	1195
II	0.045	0.398	26.0	1.135	74.0	1–78.7	1348	1190
III	0.110	0.936	61.5	0.597	38.5	1–45.1	773	1295
IV	0.150	1.196	78.1	0.337	21.9	1–28.3	485	1440
V	0.350	1.533	100.0	0	0	1–10–8	185	—

The application of this method to immune rabbit serum is rendered somewhat difficult by the fact that the protective antibody is not completely precipitated by the capsular polysaccharide as now prepared. For this reason the apparent number of protective units per mg. of precipitable nitrogen in the supernatant of precipitate VI is infinitely high. This same factor tends to distort somewhat the values in all preceding supernatants. By the determination of agglutinin nitrogen by the method of Heidelberger and Kabat (7) it has been found that at least 6 per cent of the specific antibody is not precipitated by the polysaccharide preparation used. This amount corresponds quite well with the amount of protective substances remaining in the supernatant solution.

TABLE IV

Analyses of Protective Potencies of Various Fractions of the Specifically Precipitable Antibody of Type I Antipneumococcus Rabbit Serum

Fraction		Position in precipitable range	Specific nitrogen content of fraction (by difference from Table III)	Protective units in fraction (by difference from Table III)	Protective ratios: Units per mg. specific N_2
Designation	Reference supernatant designation				
		per cent			
1	I-II	0 -26.0	0.398	482	1210
2	II-III	26.0-61.5	0.538	575	1170
3	III-IV	61.5-78.1	0.260	288	1105
4	IV-V	78.1-100.0	0.337	300	890



TEXT-FIG. 2. Protective potencies of various fractions of the specifically precipitable antibody of Type I antipneumococcus rabbit serum.

This apparent defect is, however, eliminated by translation of the data, as in the case of horse serum, into terms of protective ratios for each fraction. These calculations are shown in Table IV and presented graphically in Text-fig. 2.

From these data it will be noted that contrary to the observations with horse serum the antibody first precipitated from rabbit serum has a high protective ratio. In fact this protective value remains high throughout almost the entire range. There is a slight diminution with that antibody which is last precipitated.

The simplest conception which served to describe the results with antipneumococcus rabbit serum is that there are at most only two substances which have all the properties of specific antibodies and that both possess a relatively high protective ratio. In comparison with antipneumococcus horse serum the differences are slight. These experiments furnish additional evidence as to the marked contrasting properies of the immune sera from the two animal species.

The calculations presented have been based on determinations as to the amount of antibody precipitated, that remaining after precipitation being estimated by difference. In each instance, however, the amount of precipitable antibody in the supernatant fluids was actually determined. Since the determined residual antibody showed a quantitative parallelism with the amount estimated by difference, the calculated protective ratios are so closely parallel to those reported that they need not be detailed.

DISCUSSION AND SUMMARY

The generally held view has been that in any immune serum only a single antibody would be induced by and react with a single antigen. Were this true the various manifestations of antibody activity should show a quantitative parallelism. It has already been shown (1), however, that with antipneumococcus horse serum the mouse protective potency does not parallel the maximum amount of specifically precipitable protein except within certain well defined groups of antisera. The simplest explanation of this situation is that different horses form antibodies differing in specific protective capacity, but from our studies it seems probable that in any immune serum there may occur a mixture of antibodies which, while directed against the same antigen, possess different protective capacities, different avidities, etc. It would now appear that this latter hypothesis is the more tenable since the experiments here reported indicate the existence of antibodies of various protective potencies in horse antisera. It would not be unreasonable to hold that the antibodies of a single serum represent a series of substances with varying properties. On the basis of the present immunological fractionation experiments, the following deductions seem permissible.

1. Antipneumococcus horse sera must contain at least three, possibly many, antibody substances which react with the capsular polysaccharide. These are: (*a*) A substance which precipitates upon the addition of a relatively small amount of polysaccharide. This antibody possesses a low protective potency. (*b*) A substance which is precipitated with intermediate amounts of polysaccharide and which possesses an extremely high protective value. (*c*) A third substance which is precipitated only with the addition of relatively large amounts of polysaccharide. The protective value of this antibody is very low It may represent a degraded form.

2. With antipneumococcus rabbit serum the situation is somewhat simpler. This is in accord with the fact that with Type I antipneumococcus sera from this species there is a direct proportionality between the amount of specifically precipitable protein and the protective potency of the serum (1). The results with antipneumococcus rabbit serum indicate the existence of at least two antibody substances: (*a*) An antibody with high protective value which makes up the greater proportion of the total content. (*b*) A second substance which is precipitated only upon the addition of relatively large amounts of capsular polysaccharide. The existence of this second antibody is not clearly demonstrated by the present findings but the lower protective ratios obtained as greater amounts of antibody are removed probably indicate its existence. This may also represent degraded material.

The observations on the antibodies of both horse and rabbit antisera will be supported by experiments with immunochemical fractionation which will be reported in a subsequent paper.

CONCLUSIONS

1. In an effort to explain a certain lack of parallelism between the antibody content and the protective values of antipneumococcus horse sera, experiments have been carried out in which determined propor-

tions of the specific antibody have been removed from sera and the protective values of the residuum determined.

2. These experiments with immunological fractionation have revealed that the specific antibodies of immune sera do not possess uniform mouse protective potencies. The inference is that there may be several different substances in immune sera, each dominantly specific, yet exhibiting marked differences in avidity and in protective value.

BIBLIOGRAPHY

1. Goodner, K., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1937, **66**, 413.
2. Goodner, K., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1936, **64**, 377.
3. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **61**, 559.
4. Kirkbride, M. B., Hendry, J. L., and Murdick, P. P., *Am. J. Hyg.*, 1936, **23**, 187.
5. Muench, H., cited by Lloyd, W., Theiler, M., and Ricci, N. I., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1936, **29**, 481.
6. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1929, **50**, 809; 1935, **61**, 559, 563.
7. Heidelberger, M., and Kabat, E. A., *J. Exp. Med.*, 1937, **65**, 885.

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PROPERTIES OF THE TYPE SPECIFIC PROTEINS OF ANTIPNEUMOCOCCUS SERA

III. IMMUNOCHEMICAL FRACTIONATION OF TYPE I ANTIPNEUMOCOCCUS HORSE AND RABBIT SERA

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The immediately preceding paper of this series dealt with experiments designed to determine if all type specific antibodies of antipneumococcus serum possessed equivalent mouse protective values (1). The results obtained by comparison of the amounts of antibody protein with the protective potencies of various fractions precipitated by the addition of capsular polysaccharide furnished evidence that in antipneumococcus horse serum there exist at least three, and probably more, substances capable of reacting with the specific carbohydrate. With antipneumococcus rabbit serum evidence of marked differences in the specifically reactive antibodies is not so evident.

Although the results of these experiments were quite definite it seemed desirable to confirm the findings by some independent procedure. The most convincing confirmation would, of course, be the actual isolation of these various antibody substances.

The present paper reports the results of attempts to isolate the specific antibodies of Type I antipneumococcus sera. The method employed was immunochemical rather than purely chemical, since it was obviously important to obtain antibodies specifically directed against the capsular polysaccharide and as free as possible from other protein substances in the serum possessing properties chemically similar, but immunologically unrelated to those which characterize the specific antibody.

EXPERIMENTAL

Materials and Methods.—The general methods used for the determination of specifically precipitable nitrogen and protective potencies have been detailed in the first paper of this series (2). The methods employed in the various lipid analyses have also been reported (3).

Immunochemical Fractionation of Type I Antipneumococcus Horse Serum

After several other methods had been tried it was found that the so called dissociation method of Heidelberger and Kendall (4) offered the greatest promise. This method is selective since it involves recovery of antibodies from the immune precipitate formed by the addition of capsular polysaccharide to the homologous immune serum. The precipitate thus formed is washed and then extracted with hypertonic salt solution. As a result of this treatment some free antibody appears in the solution. These precipitates were formed on the antibody excess side of the equivalence zone since in the region of excess polysaccharide the bound antibodies are not freed upon extraction with hypertonic salt solution.

Heidelberger and Kendall using a serum containing both Type I and Type II antibodies found, however, that the immune precipitate formed upon the addition of Type II polysaccharide yielded both Type I and Type II antibodies upon extraction. According to now prevalent views the Type I antibody could scarcely have been precipitated as a result of true antigen-antibody union, since the addition of Type II polysaccharide to a Type I antiserum does not give a visible precipitate. Since this experiment was carried out with a polyvalent serum two explanations seemed possible. First, it might be that an animal immunized to two antigens may form antibodies which in part at least are polyvalent, that is, each antibody molecule might contain groupings which would react with both antigens. Secondly, it might be that each antibody in polyvalent serum is strictly monovalent but that those of the heterologous type become occluded in the immune precipitate as a result of adsorption.

The possibility of the participation of adsorptive phenomena suggested that the rôle of temperature be investigated. Consequently an experiment was arranged so that immune precipitates were formed at various temperatures from mixtures of monovalent sera and from a polyvalent serum.

Immune precipitates were prepared from these sera by the addition of Type I pneumococcus capsular polysaccharide at two contrasting temperatures, 4° and

37°C. In each instance the amount of polysaccharide was insufficient to fully saturate the antibody in the serum. After 1 hour the tubes were centrifuged and the precipitates washed twice with saline at the respective temperatures. To each washed precipitate was then added 1.0 cc. of 6.4 per cent NaCl solution and the precipitates were thoroughly dispersed in this fluid. All tubes were incubated 1 hour at 37°C. and were then centrifuged and the supernatant fluid removed and diluted with three volumes of distilled water so as to bring the salt concentration to 1.6 per cent. To portions of these supernatants were then added various polysaccharides in order to detect the presence of homologous and heterologous antibodies.

TABLE I

Effect of Temperature upon the Absolute Specificity of Immune Precipitation

Serum	Polysaccharide added	Reaction	Mixtures incubated 1 hr.; washed twice with saline at respective temperature. 1 cc. 6.4 per cent NaCl added to each tube and all incubated 1 hr. at 37°C. Centrifuged. Supernatant diluted 1-4 with distilled water. Extract tested against following polysaccharides:			
			Type I	Type II	C	Saline
		°C.				
Mixed Type I and Type II........	Type I	37	++++	−		−
Same.............	" "	4	++++	++		−
Polyvalent Types I and II..........	" "	37	++++	−	−	−
Same.............	" "	4	++++	++	+	−

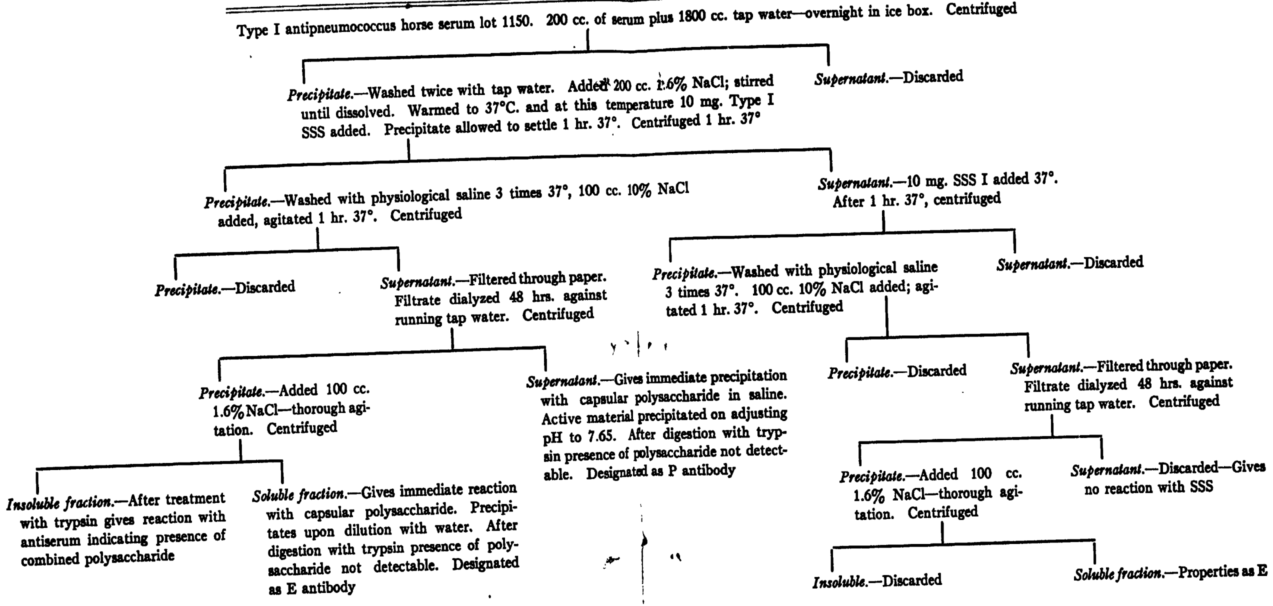
The results of this experiment are shown in Table I. These data demonstrate that at 4°C. certain amounts of heterologous as well as homologous antibodies had been carried down in the precipitate formed upon the addition of Type I capsular polysaccharide. On the other hand those reactions carried out at 37°C. appear to have been entirely specific since the heterologous antibodies were not extracted from these immune precipitates.

Because of the relationship to temperature it seems reasonable to infer that heterologous antibodies may become involved in the formation of immune precipitates as a result of adsorption. Furthermore these results suggest that antibodies are relatively monovalent even though formed at the same time as a result of two specific stimuli.

With the knowledge that only the specific antibodies are precipi-

TABLE II

Protocol of Experiment for the Selective Isolation of the Type Specific Antibodies of Antipneumococcus Horse Serum



- Type I antipneumococcus horse serum lot 1150. 200 cc. of serum plus 1800 cc. tap water—overnight in ice box. Centrifuged
  - *Precipitate.*—Washed twice with tap water. Added 200 cc. 1.6% NaCl; stirred until dissolved. Warmed to 37°C. and at this temperature 10 mg. Type I SSS added. Precipitate allowed to settle 1 hr. 37°. Centrifuged 1 hr. 37°
    - *Precipitate.*—Washed with physiological saline 3 times 37°, 100 cc. 10% NaCl added, agitated 1 hr. 37°. Centrifuged
      - *Precipitate.*—Discarded
      - *Supernatant.*—Filtered through paper. Filtrate dialyzed 48 hrs. against running tap water. Centrifuged
        - *Precipitate.*—Added 100 cc. 1.6% NaCl—thorough agitation. Centrifuged
          - *Insoluble fraction.*—After treatment with trypsin gives reaction with antiserum indicating presence of combined polysaccharide
          - *Soluble fraction.*—Gives immediate reaction with capsular polysaccharide. Precipitates upon dilution with water. After digestion with trypsin presence of polysaccharide not detectable. Designated as E antibody
        - *Supernatant.*—Gives immediate precipitation with capsular polysaccharide in saline. Active material precipitated on adjusting pH to 7.65. After digestion with trypsin presence of polysaccharide not detectable. Designated as P antibody
    - *Supernatant.*—10 mg. SSS I added 37°. After 1 hr. 37°, centrifuged
      - *Precipitate.*—Washed with physiological saline 3 times 37°. 100 cc. 10% NaCl added; agitated 1 hr. 37°. Centrifuged
        - *Precipitate.*—Discarded
        - *Supernatant.*—Filtered through paper. Filtrate dialyzed 48 hrs. against running tap water. Centrifuged
          - *Precipitate.*—Added 100 cc. 1.6% NaCl—thorough agitation. Centrifuged
            - *Insoluble.*—Discarded
            - *Soluble fraction.*—Properties as E
          - *Supernatant.*—Discarded—Gives no reaction with SSS
      - *Supernatant.*—Discarded
  - *Supernatant.*—Discarded

tated at 37°C. and that release of antibody can only be obtained on the antibody excess side of the equivalence zone, a method was available for the selective isolation of capsular carbohydrate antibodies from antipneumococcus sera.

The procedure adopted with antipneumococcus horse serum can best be presented in the form of a protocol as in Table II. The serum is first diluted with water in order to precipitate the so called water-insoluble globulins. The water-insoluble fraction contains most of the type specific antibodies and by this initial procedure a certain degree of purification is effected by elimination of a considerable amount of inert material. The protein precipitate is then dissolved in 1.6 per cent NaCl and warmed to 37°C. At this temperature a small amount of the capsular polysaccharide is added and the resulting precipitate allowed to settle for 1 hour. After this time the supernatant fluid is removed and the precipitate washed three times with warmed saline by centrifugation. To the precipitate is then added an appropriate amount of 10 per cent NaCl and the contents are agitated for 1 hour. The supernatant fluid is then filtered through paper and dialyzed 48 hours against running tap water. In the meantime to the supernatant resulting after the first immune precipitation an additional amount of specific polysaccharide was added and the resulting precipitate extracted in exactly the same manner. Addition of small amounts of polysaccharide have been used rather than one larger amount so as to obtain precipitates representing different points in the scale of avidity.

From the hypertonic salt extracts of these immune precipitates, as well as those of all experiments, three separate fractions were recovered. The first fraction, usually present in all extracts, consists of material which precipitated during dialysis and was not then soluble in 1.6 per cent salt solution and was only sparingly soluble in more concentrated salt solutions. No evidence was found of the presence of free polysaccharide in this material, although after heating to denature the protein and treating the coagulum for a few minutes with trypsin at pH 8.2 a strong reaction was obtained with immune serum indicating that the enzymatic digestion had released free polysaccharide. This fraction is regarded as a combined polysaccharide-antibody complex.

A second fraction, protein in nature, which also precipitated as a result of dialysis was completely soluble in 1.6 per cent salt solution. The resulting viscous opalescent solution gave a strong reaction with capsular polysaccharide and as will be shown below had excellent mouse protective properties. This substance is designated as antibody E.

The hypertonic salt solution extract of the precipitate obtained upon the first addition of the polysaccharide to the antibody solution contained a third material which did not precipitate upon dialysis but which nevertheless gave strong reactions with added polysaccharide. This protein was water-soluble except in the neighborhood of pH 7.65. If brought to this point the protein associated with antibody activity separated out slowly as a fine white precipitate. Solutions of this material also possessed the property of protecting mice. This form of antibody, designated as P, is usually obtained only from the first precipitate formed on the addition of the polysaccharide.

This method of fractionation has been repeatedly carried out with various lots of immune horse sera and has invariably yielded these two forms of type specific antibody. The various determined properties of the two antibody solutions may be summarized as follows:

Solubilities.—The E antibody is water-insoluble and is precipitated from solution by third saturation with ammonium sulfate. In this respect it resembles an euglobulin. The P antibody is water-soluble except at its isoelectric point, approximately pH 7.6. It is not precipitated until half saturation with ammonium sulfate is reached. It thus resembles a pseudoglobulin and in these properties it appears to be identical with the material described by Goebel and Chow (5).

Avidities.—If solutions of these two antibodies are diluted to the same protein concentration and similar amounts of capsular polysaccharide added, care being taken to avoid convection currents, it is found that the P antibody precipitates much more rapidly. In this respect it may be thought of as possessing the greater avidity, the latter term having been generally used to indicate rate of precipitation rather than rate of union. This finding is supported by the fact of isolation of the P form from the precipitate formed on the addition to the serum of the smallest amount of polysaccharide.

Combining Ratios.—In the range of antibody excess the following mean combining ratios have been determined.

E: 2.6 mg. nitrogen per mg. capsular polysaccharide
P: 23.4 " " " " " "

These combining ratios are consistent with the observations as to the respective avidities of the two forms of antibody.

Heterologous Reactivity.—Type I antipneumococcus horse serum gives few heterologous cross reactions. Goebel and Hotchkiss (6) have shown, however, that if galacturonic acid (a constituent of the capsular polysaccharide) is attached to a protein, the resultant conjugated antigen gives a precipitin reaction when added to Type I horse serum. This reaction is not obtained with Type I antipneumococcus rabbit serum nor is it possible with this antigen to remove all of the type specific antibody from the immune horse serum. It has been found that this antigen gives a precipitin reaction with solutions of the P antibody but does not react with the E antibody.

Specific Precipitability.—As a means of determining the relative state of immunological purity of the two forms of antibody isolated by the methods described, analyses have been carried out to determine the specific precipitability of various preparations. Results of analyses on two separate preparations of the E and P antibodies are shown in Table III.

TABLE III

Specific Precipitability of Antibody Preparations

Form	E antibody		P antibody	
Preparation No.	1	2	1	2
Total nitrogen, *mg. per cc.*	0.154	0.183	0.141	0.445
Amount of capsular polysaccharide required for maximum precipitation, *mg. per cc.*	0.20	0.10	0.10	0.15
Total nitrogen in precipitate from 1.0 cc. of antibody preparation, *mg.*	0.161	0.187	0.130	0.408
Less correction for nitrogen of polysaccharide in immune precipitate (estimated on basis of 75 per cent of that added), *mg.*	0.008	0.004	0.004	0.006
Net protein nitrogen in precipitate, *mg. per cc.*	0.153	0.183	0.126	0.402
Specific precipitability, *per cent*	99.4	100.0	89.3	90.3

It will be noted that the determined figures have been corrected for the amount of polysaccharide nitrogen probably present in the precipitate. It is assumed on the basis of results with whole serum that approximately 75 per cent of the added polysaccharide is combined in the immune precipitate in the range of maximum precipitation. The figures show that preparations of the E antibody have been obtained in a state of complete precipitability with the specific polysaccharide, that is, immunological purity. With the P antibody, on the other hand, no preparation has been obtained which is completely precipitable upon the addition of the specific polysaccharide. It is not as yet certain whether these latter preparations should be regarded as impure or whether a solubility coefficient must be considered. In view of the more soluble nature of the P antibody itself it would not be surprising if the antigen-antibody complex formed from it were also more soluble.

Lipid Content.—Analyses for lipid carbon and lipid phosphorus, carried out by the method of Kirk, Page, and Van Slyke (7), gave the following information.

Antibody	Total lipid, mg. per mg. of protein	Phosphatide, per cent of total lipid
E	0.044	22.5
P	0.034	12.5

If the antibody is regarded as a protein-lipid complex containing the determined amount of lipid, one obtains the following per cent content. E, 4.22 per cent; P, 3.29 per cent. It is of interest to note that these figures are in close agreement with those obtained in previous studies on the relation of the phospholipids to the reactivity of immune sera (8).

If one assumes that the antibody proteins are built up of units of molecular weight approximating 34,500 it is easily calculated that in the E antibody there is one phosphatide molecule (m.w. 778) for every two protein units. With the P antibody the result is one molecule of phosphatide per 5 protein units. In order to determine if the phosphatide were lecithin or cephalin, the antibody preparations were analyzed for the presence of lipid-amino-nitrogen. None was detectable in either the P or the E antibody solutions. The inference is therefore that the phosphatide in both instances is lecithin.

TABLE IV

Mouse Protective Potencies of Antibody Preparations

	Antibody E Preparation 2	Antibody P Preparation 1
Dilution end-point	1–21.2	1–2.9
Mouse protective units per cc. (simultaneous titration of standard (1000 units per cc.) = 1–75)	283	39
Maximum specifically precipitable nitrogen (corrected for polysaccharide nitrogen) *mg. per cc.*	0.183	0.126
Mouse protective units per mg. of specifically precipitable nitrogen	1550	230

It cannot be absolutely stated that the specific antibodies in immune horse serum are lipoproteins although the present results as well as those previously obtained strongly support this view. Furthermore the finding that lecithin is closely associated with both the P and the E antibody confirms the results of previous qualitative tests in which it was found that the *in vitro* reactivity of lipid-extracted antipneumococcus horse serum could be restored by the addition of very small amounts of lecithin (8). This additional evidence lends further support to the view that the type specific antibodies of antipneumococcus horse serum are lecithoproteins.

Mouse Protective Potencies.—Mouse protective titrations have been carried out by the method outlined in a previous paper (2), one set of data so obtained for each antibody preparation is summarized in Table IV.

The results show marked differences in the mouse protective potencies of the two antibodies. The more avid antibody, P, possessed only 230 mouse protective units per mg. of specifically precipitable nitrogen, while E gave results indicating 1550 units per mg.

By reference to Table II and Text-fig. 1 of the previous paper (Paper II of this series (1)) it will be noted that the first antibody precipitated on immunological fractionation contained approximately 500 protective units per mg. of specifically precipitable nitrogen. In view of the present findings this fraction may be regarded as a mixture of the P and the E antibody although consisting largely of the former. The fractions designated as 3 and 4 in the preceding paper are considered to represent almost pure E antibody since the protective ratio of these fractions was practically identical with that of the isolated form.

Immunochemical Fractionation of Antipneumococcus Rabbit Serum

Somewhat similar procedures have also been applied to antipneumococcus rabbit serum but the work has not progressed to the point which warrants definitive conclusions.

Since the specific antibodies of antipneumococcus rabbit serum are not precipitated when the serum is diluted with water, it has not been possible to eliminate much of the inert material by this initial step. It has been necessary, therefore, to obtain the immune precipitates with the serum directly and in order to approach purity it has been necessary to repeat this procedure with antibody solutions. Consequently the recovery of antibody from rabbit serum has been low and the lack of material has precluded a systematic analysis. However, on the basis of the experimental data available certain tentative statements may be made.

It has not been possible to recover from immune rabbit serum a water-soluble antibody corresponding to the so called P antibody of immune horse serum. The active material recovered has been water-insoluble, thus corresponding to the E antibody of horse serum. No claim for immunological purity of this substance can be made on the basis of the few analyses performed but it appears to be at least 85 per cent precipitable by the specific capsular polysaccharide. The mouse protective potency of the E antibody of immune rabbit serum is high, approximately 1100 units per mg. of specifically precipitable nitrogen.

These tentative findings are in complete accord with the results of immunological fractionation experiments presented in the previous paper of this series (1).

SUMMARY AND DISCUSSION

The immunological fractionation of Type I antipneumococcus horse serum, reported in the preceding paper of this series, indicated the existence of at least three forms of type specific antibody (1). This is the simplest interpretation of the results although it is possible that there exists a long series of closely related forms of antibody. Immunochemical fractionation, as reported in the present paper, indicates the existence of at least two distinct forms, the third not being demonstrable by the method employed since it is precipitated only with excess antigen. The three postulated forms of antibody of antipneumococcus horse serum together with certain of their properties may be briefly summarized as follows:

1. The So Called P Antibody.—Water-soluble (except at isoelectric point pH 7.6), pseudoglobulin-like substance (probably a lecithoprotein) which is precipitated from solution by the addition of small amounts of specific polysaccharide. Its specificity is not absolute and its capacity to protect mice is low (230 units per mg. of specifically precipitable nitrogen).

2. The So Called E Antibody.—Water-insoluble, euglobulin-like substance (probably a lecithoprotein) with low specific combining ratio. This antibody has been isolated in a state of immunological purity. Its capacity to protect mice is very high.

3. A Third Antibody Form.—Chemical properties are unknown since it has not been isolated. From immunological studies its existence may be postulated. Relatively large amounts of specific polysaccharide are required for its precipitation and the present evidence indicates a very low mouse protective capacity.

With antipneumococcus rabbit serum the evidence points to the existence of not more than two antibody forms. The first comprises a large proportion of the total specific antibody and in most of its properties it corresponds to the E antibody of immune horse serum. It has a high mouse protective value. The existence of a second form of antibody (corresponding to the third of horse serum) was not conclusively demonstrated by immunological experiments, but the fact that the last fraction of antibody precipitated from rabbit serum upon the addition of the specific polysaccharide had a somewhat lower protective value may indicate that in the rabbit serum as in that from the horse there is a final form, possibly representing degraded material, with lower protective value. These experiments offer evidence that the injection of a complex antigen, the Pneumococcus, gives rise in certain animals to more than one form of antibody reactive with the type specific capsular polysaccharide. No explanation for this fact is now available.

It is possible that in immune serum some form of equilibrium may exist between the various antibody forms, this equilibrium being conditioned by secondary components, as for example lipids. This last thesis would require that the two major antibody forms in antipneumococcus horse sera should be interconvertable, that is, it should be possible to derive the P form from the E. As yet there is no experimental justification for this point of view.

CONCLUSIONS

Immunological and immunochemical fractionation of Type I antipneumococcus horse and rabbit sera have demonstrated the existence of several forms of immune protein, each fraction behaving as a type specific antibody, but differing from the others in chemical and immunological properties.

BIBLIOGRAPHY

1. Goodner, K., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1937, **66**, 425.
2. Goodner, K., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1937, **66**, 413.
3. Horsfall, F. L., Jr., and Goodner, K., *J. Exp. Med.*, 1936, **64**, 583.
4. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1936, **64**, 161.
5. Goebel, W. F., and Chow, B. F., *J. Exp. Med.*, 1935, **62**, 179.
6. Goebel, W. F., and Hotchkiss, R., *J. Exp. Med.*, 1937, **66**, 191.
7. Kirk, E., Page, I. H., and Van Slyke, D. D., *J. Biol. Chem.*, 1934, **106**, 203.
8. Horsfall, F. L., Jr., and Goodner, K., *J. Exp. Med.*, 1935, **62**, 485.

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CHEMO-IMMUNOLOGICAL STUDIES ON THE SOLUBLE SPECIFIC SUBSTANCE OF PNEUMOCOCCUS

III. THE STRUCTURE OF THE ALDOBIONIC ACID FROM THE TYPE III POLYSACCHARIDE

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It has been shown in studies from this laboratory that the immunological properties of carbohydrates are determined by their chemical structure and configuration. When the specific capsular polysaccharides of pneumococcus Types III and VIII are hydrolyzed with mineral acid, there appears in the hydrolysate an aldobionic acid, a glucose-glucuronide (1, 2). The Type III polysaccharide is built up entirely of molecules of this disaccharide acid, while the Type VIII carbohydrate contains additional glucose residues as well. It has been suggested that this aldobionic acid is the chemical unit which accounts for the serological cross-reactions exhibited by these two polysaccharides in antipneumococcus horse sera. Accordingly it has become desirable to ascertain the constitution of the glucose-glucuronide common to the capsular polysaccharides of Types III and VIII. The present communication reports the results of an investigation to elucidate the structure of this substance.

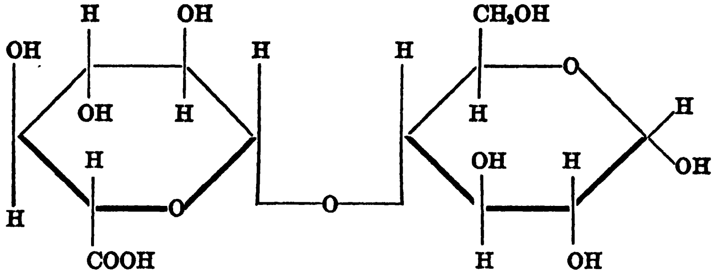
The purified aldobionic acid was methylated with dimethyl sulfate and alkali followed by treatment with methyl iodide and silver oxide, yielding a crystalline hexamethyl-β-methylglycoside methyl ester and a syrupy isomer. The former substance, when saponified with dilute alkali, was converted into crystalline hexamethyl aldobionic acid β-methylglycoside. The latter free acid derivative was hydrolyzed by boiling with 1.5 N hydrochloric acid for several hours, giving 2,3,6-trimethylglucose and 2,3,4-trimethylglucuronic acid in good yields. The glucose derivative was crystalline and identical in all of its properties with an authentic sample of 2,3,6-trimethylglucose prepared from octamethyllactose (3). The trimethylglucuronic acid was identified by its specific rotation and analysis, and by conversion into crystalline 2,3,4-trimethyl-β-methylglucuronide, identical in properties with a sample of the same substance prepared by direct methylation of pure glucuronic acid (4).

Isolation of a trimethylglucuronic acid having a 1,5 oxygen bridge is ample demonstration that the aldobionic acid itself is a glucurono*pyranoside*. A stable pyranose configuration is to be expected in view of the fact that the aldobionic acid resists prolonged treatment with boiling N sulfuric acid during its preparation from the polysaccharides. The position of the oxygen bridge in the glucose portion of the disaccharide may not be so readily inferred. The occurrence of 2,3,6-trimethylglucose among the hydrolysis products of a completely methylated carbohydrate usually indicates a glycoside linkage between 1 sugar molecule and the 4th carbon atom of a gluco*pyranose* residue. However, the possibility remains that the biose linkage in the aldobionic acid could involve the 5th carbon atom of a gluco*furanose* residue. That this is not the case has been shown by a study of the rate of hydrolysis of the hexamethyl-β-methylglycoside derivative in N hydrochloric acid at 100°, as determined by the liberation of reducing sugar. The apparent velocity constant calculated for a unimolecular reaction by the method of Guggenheim (5) is 10×10^{-6} min.$^{-1}$ computed for 0.01 N hydrochloric acid. This value is of the same order as that found by Haworth and Hirst (6) for tetramethyl methylglucoside ($k = 23 \times 10^{-6}$ min.$^{-1}$ in natural logarithms), and is very different from the values obtained for the hydrolysis of furanosides, which are 100 or more times larger. Although the aldobionic acid derivative contains two different glycosidic linkages which hydrolyze simultaneously, it is obvious that if one of them were a furanoside the apparent velocity constant would be much greater than the value obtained. The aldobionic acid can definitely be formulated, therefore, as a glucopyranose-4-glucuronopyranoside.

Thus far it is not possible to state whether the biose linkage has the α or β configuration; that is, whether the aldobionic acid is of the maltose or cellobiose type. However, certain information derived from molecular rotation data indicates clearly the occurrence of the β linkage. The aldobionic acid may be regarded, therefore, as cellobiouronic acid.

The molecular rotations in chloroform of the known acetylated derivatives of glucuronic acid methyl ester differ from those of the corresponding acetylated glucose derivatives of the same configuration by an approximately constant amount. If, in pentaacetylglucose, A represents the molecular rotation due to the glycosidic carbon atom bearing an acetyl group, and G the rotation due to the remainder of the molecule, the molecular rotation of α-pentaacetylglucose is (7) $G + A = 39{,}600°$ (*a*). Since the rotation due to the glycosidic carbon atom is approximately the same for all sugar acetates of the *d* series, the molecular rotation for α-tetraacetylglucuronic acid methyl ester is (8) $Glr + A = 36{,}800°$ (*b*), in which *Glr* symbolizes the molecular rotation due to the remaining 5 carbon atoms of the uronic acid molecule. By subtracting (*b*) from (*a*), one obtains $G - Glr = 2800°$ (*c*). Similarly, for β-acetates of the two sugars, according to the data from the literature (7, 8), $G - Glr = 1480° - 3300° = -1820°$ (*d*). From the work of Hudson (7, 9), and Freudenberg (10) one would expect, in the case of disaccharides, the difference $G - Glr$ to remain approximately constant whenever the acetylated glucose or glucuronic ester is linked, with the same configuration, to identical constituents. That this is true is evident from a comparison of the molecular rotations of the acetates of gentiobiose (glucose-6-β-glucoside) and gentiobiuronic acid (glucose-6-β-glucuronide). If the molecular rotation of α-heptaacetylgentiobiuronic acid methyl ester (11) is subtracted from that of α-octaacetylgentiobiose (7) one obtains $G - Glr = 35{,}500° - 32{,}200° = 3300°$ (*e*). From the corresponding β-heptaacetyl ester and β-octaacetate, $G - Glr = -3590° - (-)7300° = 3710°$(*f*).

By using the average value of equations (*c*) to (*f*), $G - Glr = 2000°$, one can calculate the molecular rotations of the hypothetical α-heptaacetyl methyl ester of glucose-4-(α and β)-glucuronides. By subtracting 2000° from the value for α-octaacetylmaltose (glucose-4-α-glucoside) we have $83{,}000° - 2000° = 81{,}000°$ as the expected molecular rotation of the α-heptaacetyl methyl ester of glucose-4-α-glucuronide. Similarly from α-octaacetylcellobiose (glucose-4-β-glucoside) one may calculate the value 27,800° − 2000° = 25,800° for the α-heptaacetyl methyl ester of glucose-4-β-glucuronide. The α-heptaacetyl methyl ester of the aldobionic acid from pneumococcus has a molecular rotation of 27,100° (2) and is obviously the β-glucuronide. The parent aldobionic acid is accordingly the glucose-4-β-glucuronide shown in the accompanying diagram.



The close relationship of this disaccharide to cellobiose is of interest, since it suggests a relationship of the pneumococcus Type III and VIII polysaccharides to cellulose. The manner in which the aldobionic acid molecules themselves are linked to each other and to glucose in the polysaccharides is not known at present. If, however, the uronic acid residue is linked as a β-glycoside on the 4th carbon atom, as seems quite possible, the polysaccharides may be visualized as long cellulose-like chains in which certain of the sugar residues have been oxidized to glucuronic acid. The structure of these two polysaccharides is being investigated further in this laboratory.

EXPERIMENTAL

Preparation of Brucine Aldobionate—The barium salt of the aldobionic acid was prepared from the Type III pneumococcus polysaccharide as previously described (1, 2). 12 gm. of crude salt were dissolved in 500 cc. of H_2O and treated with a slight excess of aqueous brucine sulfate at 60°. After centrifugation to remove barium sulfate, treatment with charcoal, and concentration *in vacuo*, the brucine salt crystallized. About 10 gm. were recovered, and after several recrystallizations from 75 per cent acetone, 7.2 gm. of pure salt were obtained, melting at 187–188° (uncorrected) with decomposition. The material appears to contain 2 molecules of water of crystallization. (Calculated, H_2O 4.58; found, 4.47.) After drying at 100°, the following analytical constants were observed.

$[\alpha]_D^{27} = -14.7°$ (at 3 min.) changing to $-15.7°$ (water solution, $c = 1.5$)
Analysis—$C_{11}H_{19}O_{10}(COOH)(C_{23}H_{26}N_2O_4)$

Calculated.	C 56.0,	H 6.18,	N 3.73
Found.	" 56.1,	" 6.41,	" 3.83

Heptaacetyl Methyl Ester of Aldobionic Acid—In order to show that the crystalline brucine salt is a derivative of the amorphous aldobionic acid previously studied (2) a portion was converted into the heptaacetyl methyl ester. 1 cc. of a solution containing 150 mg. of sodium aldobionate was acidified with 0.1 cc. of 6 N hydrochloric acid and dissolved in 150 cc. of absolute methanol. The solution was treated with an excess of diazomethane in ether, neutralized with calcium carbonate, filtered, and concentrated *in vacuo*. The residue was acetylated with pyridine and acetic anhydride. The crude product (135 mg.) gave after several recrystallizations a small amount of silky needles melting at 249° (uncorrected). The melting point was not depressed when these crystals were mixed with a sample of the heptaacetyl methyl ester prepared from the aldobionic acid of Type III and VIII polysaccharides as previously described (2). The mother liquors gave a crude crystalline product melting at about 205° and having a specific rotation lower than that of the crystals melting at 249°. It seems certain, therefore, that the substance melting at the higher temperature is the α-heptaacetate and not the β form as originally suggested.

Methylation of Aldobionic Acid—6.16 gm. of the pure brucine salt of the aldobionic acid (corresponding to 2.79 gm. of aldobionic acid) were dissolved in 200 cc. of water. An excess of sodium hydroxide was added and the precipitated brucine filtered off. The filtrate and washings after extraction with chloroform were neutralized with sulfuric acid and concentrated *in vacuo* to 20 cc. 1 cc. was removed for the preparation of the heptaacetyl methyl ester as described above. The remainder was methylated at 35° and later at 50°, a total of 20 cc. of dimethyl sulfate and 45 cc. of 30 per cent sodium hydroxide being used. After heating at 100° for ½ hour the mixture was acidified and extracted with chloroform. The aqueous layer was neutralized, concentrated, and remethylated after removal of sodium sulfate with alcohol. The combined chloroform extracts from the first and second methylations yielded 3 gm. of viscous syrup.

The syrup was treated with 30 cc. of methyl iodide and 10 gm. of silver oxide added in portions at 45°. The mixture when extracted with chloroform gave 2.7 gm. of a syrup. Without being submitted to distillation, the material was induced to crystallize; 1.8 gm. of crude crystalline substance were obtained. After recrystallization from ether-petroleum ether 1 gm. of prismatic needles was recovered, melting sharply at 113–113.5° (uncorrected). From the analytical constants it is obvious that the substance is the hexamethyl-β-methylglycoside methyl ester of the aldobionic acid.

$[\alpha]_D^{26} = -32.2°$ (water, $c = 1.3$)
Analysis—$C_{11}H_{12}O_3(OCH_3)_7(COOCH_3)$
Calculated. C 51.3, H 7.75, OCH_3 53.0, $COOCH_3$ 12.60
Found. " 51.7, " 7.93, " 50.9, " 12.56

The non-crystalline fraction of the syrup had a higher specific rotation than the crystalline. The latter is assumed to be the β-methylglycoside.

Hexamethyl-β-Methylglycoside of Aldobionic Acid—0.9 gm. of the hexamethyl-β-methylglycoside methyl ester was dissolved in a slight excess of 0.1 N sodium hydroxide at 100°. The solution was acidified and extracted with chloroform. When dried and concentrated *in vacuo* the extracts left a syrupy residue which crystallized spontaneously when dissolved in absolute ether. The product was recrystallized from ether, yielding 0.75 gm. of needles melting sharply at 171.5–172° (uncorrected).

$[\alpha]_D^{26} = -32.6°$ (water, $c = 0.8$)
Analysis—$C_{11}H_{12}O_3(OCH_3)_7(COOH)$
Calculated. C 50.2, H 7.54, OCH_3 47.8, COOH 9.91
Found. " 50.5, " 7.54, " 45.6, " 9.71

Hydrolysis of Hexamethyl-β-Methylglycoside—0.79 gm. of hexamethyl aldobionic acid β-methylglycoside was dissolved in 34 cc. of 1.5 N hydrochloric acid and boiled under a reflux condenser. The optical rotation of the solution remained constant at +3.2° (observed in a 2 dm. tube) after 5 hours. The solution was neutralized with barium carbonate and evaporated to dryness *in vacuo*. The residue was extracted repeatedly with ether. From the ether solution was obtained 0.26 gm. of crystalline trimethylglucose, which on recrystallization yielded characteristic long needles melting at 113.5–114° (uncorrected). A mixture of the substance with an authentic sample of 2,3,6-trimethylglucose (m.p. 113–114°) prepared from octamethyllactose (3), melted likewise at 113.5–114°. Its specific rotation in water ($c = 0.3$) was $[\alpha]_D^{28} = +96.6°$ (5 minutes after solution), changing to a final constant value of +70.7°. Irvine and Hirst (12) give +90.2° and 70.5° respectively as the initial and final values for 2,3,6-trimethylglucose in aqueous solution.

Analysis—$C_6H_9O_3(OCH_3)_3$. Calculated, OCH_3 41.9; found, OCH_3 41.5

The residue of dry barium salts containing the methylated uronic acid was dissolved in water, treated with 1.55 cc. of N sulfuric acid, and evaporated to dryness. Extraction of the residue with ether yielded 0.33 gm. of a pale yellow syrup, which showed the following specific rotation and analysis.

$[\alpha]_D^{27} = +54.3°$ (equilibrium value in water, $c = 0.5$)
Analysis—$C_5H_6O_2(OCH_3)_3(COOH)$. Calculated. OCH_3 39.4
Found. " 38.5

Challinor, Haworth, and Hirst (4) report the value of +58° for 2,3,4-trimethylglucuronic acid prepared in a similar way from galactose-6-β-glucuronide.

Treatment of 0.21 gm. of the syrupy trimethylglucuronic acid with dimethyl sulfate and alkali at 35° and 55° gave 0.18 gm. of methylated product. From this there was isolated 0.03 gm. of the crystalline β-methylglycoside, melting at 134–135° (uncorrected) and showing

$[\alpha]_D^{28} = -31°$ (water, $c = 0.2$)
Analysis—$C_5H_5O(OCH_3)_4(COOH)$
Calculated. OCH_3 49.6, COOH 18.0
Found. " 48.7, " 18.1

A melting point of 134–135° was observed for a mixture of this substance with 2,3,4-trimethylglucuronide prepared from pure glucuronic acid (4).

Kinetics of Hydrolysis of Hexamethyl Aldobionic Acid β-Methylglycoside—0.070 gm. of the hexamethyl-β-methylglycoside of the aldobionic acid was dissolved in 50 cc. of N hydrochloric acid and boiled under a reflux condenser. Samples were withdrawn at intervals and the amount of reducing sugar determined by the Hanes modification (13) of the Hagedorn-Jensen method. Table I shows the average volume of 0.01 N thiosulfate required for the back titration of the ferricyanide reduced by duplicate 2.0 cc. portions.

The reaction velocity constant for the hydrolysis was calculated by the method of Guggenheim (5). The apparent velocity constant at 100° is $k = 10 \times 10^{-3}$ min.$^{-1}$ for N hydrochloric acid, or assuming proportionality to the hydrogen ion concentration, $k = 10 \times 10^{-5}$ min.$^{-1}$ for 0.01 N hydrochloric acid. When recalculated on the basis of natural logarithms, the values found by Haworth and Hirst (6) for pyranosides in 0.01 N acid at 100° range from 9×10^{-5} to 70×10^{-5} min.$^{-1}$. The corresponding constants for the furanosides range from 600×10^{-5} to $11{,}000 \times 10^{-5}$.

TABLE I

Hydrolysis of Hexamethyl Aldobionic Acid β-Methylglycoside in HCl at 100°

Time, *min*.	0	30	60	120	210	360	540
0.01 N thiosulfate, *cc*.	12.21	11.11	10.27	9.08	8.41	7.75	7.41

SUMMARY

The aldobionic acid from the specific capsular polysaccharides of Types III and VIII pneumococcus has been shown to be glucose-4-β-glucuronide. The relationship to cellobiose has been discussed.

BIBLIOGRAPHY

1. Heidelberger, M., and Goebel, W. F., *J. Biol. Chem.*, **74**, 613 (1927).
2. Goebel, W. F., *J. Biol. Chem.*, **110**, 391 (1935).
3. Haworth, W. N., and Leitch, G., *J. Chem. Soc.*, **113**, 195 (1918).
4. Challinor, S. W., Haworth, W. N., and Hirst, E. L., *J. Chem. Soc.*, 258 (1931).
5. Guggenheim, E. A., *Phil. Mag.*, series 7, **2**, 538 (1926).

6. Haworth, W. N., and Hirst, E. L., *J. Chem. Soc.*, 2615 (1930).
7. Hudson, C. S., *Scient. Papers Bur. Standards, U. S. Dept. Commerce, No. 533* (1926).
8. Goebel, W. F., and Babers, F. H., *J. Biol. Chem.*, **106**, 63 (1934).
9. Hudson, C. S., *J. Am. Chem. Soc.*, **52**, 1707 (1930).
10. Freudenberg, K., and Blomqvist, G., *Ber. chem. Ges.*, **68**, 2070 (1935).
11. Hotchkiss, R. D., and Goebel, W. F., *J. Biol. Chem.*, **115**, 285 (1936).
12. Irvine, J. C., and Hirst, E. L., *J. Chem. Soc.*, **121**, 1216 (1922).
13. Hanes, C. S., *Biochem. J.*, **23**, 99 (1929).

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ELEMENTARY BODIES OF VACCINIA FROM INFECTED CHORIO-ALLANTOIC MEMBRANES OF DEVELOPING CHICK EMBRYOS

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Plate 8

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Paschen bodies (1) or elementary bodies, have been observed in many types of cells in various animal species infected with vaccine virus (2). Suspensions of these minute structures in a relatively pure state have been prepared by means of differential centrifugation from infected skins of rabbits (3–5) and guinea pigs (6). The presence of large amounts of protein in emulsified organs has made it difficult in the past to obtain from such sources clean suspensions of virus elementary bodies. Infected skin, however, is peculiarly well adapted to the methods previously employed since the highly infectious dermal pulp, which results from scraping the skin (4, 5), contains less tissue proteins than do ground infected organs.

The preparation of suspensions of vaccine virus elementary bodies in a relatively pure state from infected tissues of a generic class different from that previously used would add to the existing evidence for the intimate association of the elementary body with the causal agent. Moreover, such an experiment would offer an opportunity for further observation on the soluble precipitable substances (6) that accompany the virus infection. Studies (7, 8) on the chick chorio-allantoic membrane inoculated with vaccine virus indicated that this tissue, which has a high infective titer and contains numerous elementary bodies, might be suitable material from which to obtain suspensions of relatively pure Paschen bodies. The purpose of this paper is to describe a method for preparing suspensions of elementary bodies from vaccine virus-infected chorio-allantoic membranes of developing chick embryos, and to present the results of serological studies on the elementary bodies and virus-free filtrates of infected membranes.

Materials and Methods

Chorio-Allantoic Membranes.—The chorio-allantoic membranes of 12 to 14 day chick embryos were exposed by Burnet's modification (9) of the technic of Woodruff and Goodpasture (10). Usually 0.1 cc. of a 10^{-3} dilution of virus suspension was dropped on the membrane surface; then the egg was gently rotated to insure spread of the inoculum. After incubation for 36–48 hours at 37°C., the infected portion of the chorio-allantoic membrane was removed. Occasional membranes that were contaminated with bacteria were discarded.

Virus.—Vaccine virus of the C. L. strain,[1] which had been carried for 4 years in this laboratory by passage of elementary bodies on rabbit skins, was rendered bacteriologically sterile by storage with ether. This virus served initially to infect chick membranes. Experiments were performed with the 2nd to the 27th membrane subculture of virus.

Antisera.—Antivaccinal sera were obtained from rabbits and guinea pigs after hyperimmunization with washed elementary bodies of rabbit origin. Monkeys recovered from intravenous inoculation of similar preparations of virus also yielded immune sera. Antisera against the heat-labile and heat-stable precipitable substances, respectively, of vaccinia which had been prepared in rabbits (11) were supplied by Dr. Robert F. Parker.

Infective Titers.—Infective titers were determined by intradermal injection into rabbits of serial tenfold dilutions of virus. Duplicate titrations were always performed.

Preparation of Elementary Body Suspensions

Suspensions of elementary bodies of vaccinia in a relatively pure state were prepared from infected chorio-allantoic membranes of chicks in the following manner.

The infected portions of 10–25 membranes were placed in Locke's solution immediately following removal from the eggs. After the last membrane was harvested the entire lot was rinsed in fresh Locke's solution in order to remove additional mucilaginous material adherent to the surface as well as blood which had oozed from the cut vessels of the membranes. The tissues were next individually dipped into ethyl ether to remove part of the lipid substances and were again rinsed in Locke's solution. Excess fluid having been drained from the membranes, they were placed in a mortar, minced with scissors, and ground vigorously. 10–20 cc. of dilute buffer solution, pH 7.2, (standard citric acid-disodium phosphate buffer, pH 7.2, diluted 50 times with distilled water) were added to the triturated material and the suspension was thoroughly shaken in a test tube. Sediment thrown down at 1000 R.P.M. in the International horizontal centrifuge was resuspended in a similar volume of buffer solution and recentrifuged. Supernatant fluids from these two centrifugations were pooled and again spun at 1000 R.P.M. for 10 minutes. The resultant supernatant suspension was distributed in flat pyrex tubes, that have an inside diameter of 4 mm. and a capacity of 5 cc., and run in a Swedish angle centrifuge for 1 hour at 3500 R.P.M. Fluid was poured off and saved for titration of precipitinogens. The angle sediment, which contained practically all of the elementary bodies as well as considerable amounts of tissue materials, was taken up in 5–10 cc. of dilute buffer solution, pH 8.0. 0.5 cc. of a filtered 1 per cent solution of commercial trypsin that had been freed from lipids by successive extraction with ethyl ether and petrol ether was added to the suspension; the mixture was incubated at 37°C. for a half to 1 hour. Fat-extracted trypsin was used because Pirie (12) found that the lipids in commercial trypsin—not the enzymes—inactivated vaccine virus. The digested material was then washed 3 to 7 times by sedimentation in the angle centrifuge and resuspension in dilute pH 7.2 buffer solution. After the final washing, the suspension was spun in the horizontal centrifuge for half an hour at a speed of 2500 R.P.M. in order to remove particles larger than elementary bodies.

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[1] Virus was obtained from Dr. J. Craigie in 1933.

In the manner described, suspensions of relatively pure elementary bodies were prepared by a method of differential centrifugation similar to that used by other workers (4, 5). However, one important additional procedure, namely, tryptic digestion, was employed. Suspensions of virus still contained some soluble tissue proteins after 3 washings. This was indicated by the presence of amorphous, brown, precipitate interspersed among the round, black, elementary bodies in smears stained by Morosow's technic (13). When such preparations were subjected to further washing the amorphous precipitate was reduced to a minimum. Fig. 1 is a photograph of a stained preparation made from a suspension of elementary bodies treated with trypsin and washed 7 times in all.

Protein digestion was an important step in purification since repeated washing of the sediment from the angle centrifugation with dilute buffer solution alone failed to remove adequately tissue proteins. Physiological salt solution was not employed because Craigie (14) found that vaccine virus elementary bodies in the presence of 0.85 per cent saline readily underwent spontaneous agglutination. Treatment with trypsin usually reduced the amount of the angle sediment to one-half to two-thirds of its original volume.

Only a portion of the elementary bodies present in infected membranes was secured by the method described as routine. In 3 instances, cells and debris, sedimented during preliminary horizontal centrifugations and discarded in the regular procedure, were first subjected to tryptic digestion and then to differential centrifugation. The resultant material, after repeated washing and additional enzymatic treatment, was found to be rich in elementary bodies. The infective titer, moreover, was in each case the same as that of the elementary body suspension prepared according to routine. These suspensions were not so clean as the regularly prepared ones, as evidenced by the precipitated amorphous material in stained smears. Such suspensions were not satisfactory for use in immunological tests because marked spontaneous agglutination occurred.

Infective Titer of Elementary Body Preparations

The most important single fact that has led to an acceptance of the close relationship between elementary bodies of vaccinia and the agent responsible for the disease is the correlation of the infective titer of suspensions with their elementary body content. The virus accompanies the elementary bodies from rabbit dermal pulp (3–5) even when filtered (15); the bodies increase in tissue culture with multiplication of the virus (16); and a definite numerical relationship exists between the bodies and a single infectious dose (17); moreover, materials freed from elementary bodies are not infectious (4, 5).

Additional evidence for the idea that elementary bodies represent the virus itself or are closely associated with the virus was brought out in the present set of experiments. The infectivity of membrane material was not appreciably altered by tryptic digestion and repeated washing; the final preparation of elementary bodies was as infectious as the original membrane emulsion. Experiment 5, summarized in Table I, is illustrative of the data supporting this statement. After the removal of coarse particles by preliminary centrifugation, the membrane emulsion had an infective titer of 10^{-6}. The material sedimented in the angle centrifuge was digested with trypsin and washed 5 times; this represents a dilution of the original angle sediment of the order of 1×10^{-4}. At this stage the infective titer was 10^{-7}. Further washing diluted the first angle sediment to approximately 1×10^{-6} times its original volume; nevertheless, the titer remained 10^{-7}. Two factors operated to increase the titer of the original emul-

TABLE I

Infectivity of Suspension of Elementary Bodies of Vaccinia

Experiment 5	Day	Treatment	Volume of suspension	Dilution of 1st angle sediment	Infectivity of suspension						
					10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
			cc.								
21 membranes infected with 12th passage virus	1	Membrane emulsion after horizontal centrifugation	22	0	++++	++++	++++	+++	++	±	
	2	Washed total of 5 times. Treated with trypsin after 2nd sedimentation	5	1 × 10^{-4}							
	4	Stored at 3°C.	"	" " "	++++	++++	++++	+++	++	+	±
		Washed 2 additional times (total 7)	"	1 × 10^{-6}							
	11	Stored at 3°C.	"	" " "	++++	++++	++++	+++	++	+	

TABLE II

Agglutination of Elementary Bodies of Vaccinia

Suspension	Antivaccinal serum	Dilution of serum										Type of agglutination
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1. 1280	1:2560	
Chick E.B. No. 9	Rabbit 6022			+++	+++	+++	++	++	++	+		Granular
Rabbit E.B. No. 7660	" "			+++	+++	+++	+++	+++	++	+	−	Large flocks
Chick E.B. No. 8	Guinea pig 5972	−	±	+	++	+	±					Granular
Chick E.B. No. 9	" " "	+	++	++	++	−	−	−	−			"
Rabbit E.B. No. 7078	" " "	+++	+++	+++	+	+	+	−	−			Large flocks

E. B. = elementary bodies of vaccinia.

sion. Tryptic digestion probably liberated virus from cell particles and from cells killed by immersion of the membranes in ether, for, as observed in another experiment, tryptic digestion alone raised the titer of a crude membrane emulsion from 10^{-5} to 10^{-6}. The second factor was the decrease in volume of suspending fluid; the particulate matter originally present in 22 cc. was later taken up in only 5 cc. of fluid.

Decrease in the number of elementary bodies and in the infective titer occurred to a certain extent during the manipulation incident to purification of elementary body suspensions. This was apparent when relatively few infected membranes constituted the source of virus, *e.g.*, in Experiment 3, in which 8 membranes were employed. The titer following tryptic digestion, and before washing, was 10^{-6}; after 4 washings, which diluted the original angle sediment approximately 1×10^{-7} times, the titer had fallen to 10^{-5}. Another source of loss was the thin layer of elementary bodies that always adhered to the walls of the glass tubes. Moreover, the small fraction of elementary bodies which had been sedimented along the outer wall of the centrifuge tube, but which had not yet been thrown to the bottom, was often lost in pouring off the supernatant fluid. Finally, the last horizontal centrifugation also contributed to the losses, because in several instances, the sediment which resulted from this procedure was found to be rich in elementary bodies, when stained by Morosow's technic, and had an infectivity equal or almost equal to that of the suspension prepared according to routine. Nevertheless, the final horizontal centrifugation was useful, notwithstanding the waste that accompanied it, because debris and clumped elementary bodies were removed.

Agglutination of Elementary Bodies

Agglutination of elementary bodies (18, 3) by antivaccinal serum was another significant step in establishing the importance of these structures in the disease. It seemed worth while to determine whether or not elementary bodies prepared from hosts of different generic classes were equally agglutinable with a given immune serum.

Agglutination Reaction.—Agglutination of elementary bodies was carried out in the manner described by Craigie (4) and Parker and Rivers (5). Dilutions of immune sera were made with freshly prepared physiological salt solution buffered to pH 7.2 by the addition of 1 cc. of standard citric acid-disodium phosphate buffer solution to each 100 cc. of saline solution. Elementary body suspensions were brought to the proper concentration for testing by the addition of dilute buffer solution, pH 7.2. 0.25 cc. of elementary body suspension was added to 0.25 cc. of diluted serum which had been placed in a pyrex tube with outside dimensions of 10 x 75 mm. Tubes were placed in a covered rack and incubated overnight at 50°C.

The data presented in Table II show that elementary bodies prepared from either chick membrane or rabbit skin are agglutinated approximately to the same extent by immune sera from two mammalian species. Hyperimmune rabbit serum, in a dilution of 1:1280, agglutinated elementary body suspension 9 obtained from membranes of chick embryos; the serum had the same titer when tested with an elementary body suspension derived from dermal vaccine pulp of rabbits. The guinea pig and monkey antivaccinal sera, although not so potent as the rabbit immune sera, respectively agglutinated chick membrane and rabbit dermal elementary bodies to an approximately equal degree. A consistent difference between the two virus suspensions was noted in the type of clumping. Elementary bodies of rabbit origin formed large loose flakes when mixed with immune sera from various species; in higher concentrations of serum the floccules settled and left a clear supernatant fluid. Chick membrane elementary bodies, however, agglutinated only in a granular form. Repeated washing tended to reduce the agglutinability of bodies; the highest titers were obtained when suspensions were washed 3 or 4 times.

Soluble Precipitable Substances in Filtrates of Infected Membranes

It has been pointed out by Sabin (19) that soluble antigens obtained from tissues infected with vaccinia might be "specific products of infection which are not derived from the virus substance" rather than products of the virus. Craigie and Wishart (6) subsequently demonstrated the immunological identity of L and S precipitinogens found in dermal pulp filtrates from infected rabbits, guinea pigs, and calves, and Ch'en (20) found a soluble antigen in tissue culture vaccine virus prepared according to the method of Li and Rivers (21) in which minced chick embryo tissue suspended in Tyrode's solution was used.

Further information regarding these soluble antigens was sought by means of precipitin tests on the virus-free filtrates from infected chick membranes.

Precipitin Reactions.—Precipitin tests were carried out on extracts of infected membranes. The supernatant fluid which resulted from the first angle centrifugation was filtered through a Seitz pad; the filter had been previously prepared by the passage of 10 cc. of broth and 2 cc. of normal rabbit serum. These filtrates were not infectious. Serial dilutions of the clear serum-colored filtrates were prepared with buffered saline solution and mixed with equal volumes of diluted immune serum, the optimal dilution of the immune serum having been determined by preliminary titration. Tubes and racks similar to those employed for agglutination tests were used; the mixtures were incubated overnight at 50°C.

Precipitin tests on Seitz filtrates of suspensions of infected membranes demonstrated the presence of heat-labile and heat-stable antigens. Results of such a test are shown in Table III. Hyperimmune rabbit serum, which contained antibodies against L and S fractions, gave a slightly higher titer with pooled rabbit dermal filtrate than with membrane filtrate. Rabbit serum that had L but no S antibody reacted equally well with chick membrane and rabbit skin filtrates. Pure anti-S rabbit serum, like the L-S serum, precipitated membrane filtrate at a slightly lower titer than rabbit filtrate.

The results of precipitin tests, which were done on all filtrates of infected membranes, were found to vary. No attempt was made to remove inhibitive substances (6) from the membrane filtrates; this may account for considerable variation in titer of different preparations. Membrane emulsions were filtered with difficulty through Seitz pads, even after removal of most of the particles by centrifugation. The absence of demonstrable precipitinogens in Experiments 6 and 9 probably depended on technical manipulation, such as extraction with ethyl ether and the use of several pads for filtering a small volume of material. There was no obvious explanation for the inability to demonstrate the presence of precipitinogens in filtrate 10; of interest also was the failure to find agglutinogens in the elementary body suspension prepared from this group of membranes which had been inoculated with the 24th membrane passage virus. This failure to demonstrate serologically active substances was not dependent on a change in the virus, because precipitinogens and agglutinogens were

TABLE III

Soluble Precipitable Substances in Filtrates of Chick Membranes Infected with Vaccine Virus

Filtrate		Type of antivaccinal rabbit serum	Dilution of filtrate								
Source	No.		1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
Chick membrane	8	L-S serum 6022, diluted 1:12		++++	++++	++++	++	+	−	−	−
Rabbit skin	Pool I	" " " " "		++++	++++	++++	++++	+++	++	+	−
Chick membrane	8	L serum 5110, diluted 1:2	++	++	++	+	+	−			
Rabbit skin	Pool I	" " " " "	++	++	+++	++	+	−			
Chick membrane	8	S serum 5199, diluted 1:4	++++	++++	+++	++	−	−	−		
Rabbit skin	Pool I	" " " " "	++++	++++	++++	+++	++	+	−		

present in lot 11 which had been infected with the 26th membrane passage.

DISCUSSION

The present observations suggest that by use of appropriate methods the infectivity of tissues from animals with vaccinia may be found, in each case, to be associated with their elementary body content. The experiments described in this paper throw no light on the idea of Craigie and Wishart (22) that there are two forms, resistant and active, of vaccine virus elementary bodies. Nor do the observations bring direct evidence to bear on Sabin's suggestion (19) regarding host production of the soluble precipitable substances. The finding of L and S precipitinogens in the filtrates of infected chick membranes by means of immune sera prepared in rabbits by repeated injection of elementary bodies from rabbits, might be considered additional circumstantial evidence that these antigens are products of the virus rather than the result of a response of the host to infection with the active agent.

SUMMARY

By a method of differential centrifugation and tryptic digestion suspensions of elementary bodies have been prepared from chorioallantoic membranes of chick embryos infected with vaccine virus. The infective titer of the final suspension of elementary bodies was usually the same as that of the original tissue emulsion. Elementary bodies from infected chick membranes were agglutinated as well by antivaccinal serum obtained from different mammalian species as were bodies prepared from inoculated rabbit skin. Seitz filtrates of infected chick material contained soluble precipitable substances of vaccinia; these filtrates and filtrates from infected rabbit skin, respectively, reacted equally well with rabbit serum which contained either L or S antibodies.

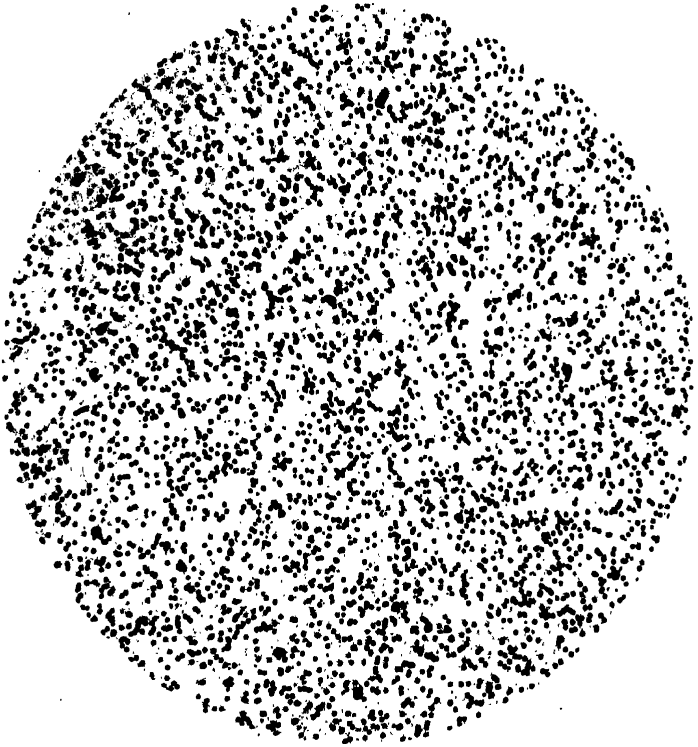
BIBLIOGRAPHY

1. Paschen, E., *Münch. med. Woch.*, 1906, **53**, 2391.
2. Haagen, E., and Kodama, M., *Zentr. Bakt.*, *1. Abt.*, *Orig.*, 1934–35, **133**, 23.
3. Ledingham, J. C. G., *Lancet*, 1931, **2**, 525.
4. Craigie, J., *Brit. J. Exp. Path.*, 1932, **13**, 259.

5. Parker, R. F., and Rivers, T. M., *J. Exp. Med.*, 1935, **62**, 65.
6. Craigie, J., and Wishart, F. O., *Tr. Roy. Soc. Canada*, 1935, **29**, 3rd series, 57.
7. Goodpasture, E. W., Woodruff, A. M., and Buddingh, G. J., *Am. J. Path.*, 1932, **8**, 271.
8. Herzberg, K., *Zentr. Bakt., 1. Abt., Orig.*, 1936, **136**, 257.
9. Burnet, F. M., *J. Path. and Bact.*, 1933, **37**, 107.
10. Woodruff, A. M., and Goodpasture, E. W., *Am. J. Path.*, 1931, **7**, 209.
11. Parker, R. F., and Rivers, T. M., *J. Exp. Med.*, 1937, **65**, 243.
12. Pirie, A., *Brit. J. Exp. Path.*, 1935, **16**, 497.
13. Morosow, M. A., *Centr. Bakt., 1. Abt., Orig.*, 1926, **100**, 385.
14. Craigie, J., and Wishart, F. O., *Brit. J. Exp. Path.*, 1934, **15**, 390.
15. Eagles, G. H., and Ledingham, J. C. G., *Lancet*, 1932, **1**, 823.
16. Nauck, E. G., and Paschen, E., *Zentr. Bakt., 1. Abt., Orig.*, 1932, **124**, 91.
17. Parker, R. F., and Rivers, T. M., *J. Exp. Med.*, 1936, **64**, 439.
18. Nocht, B., and Paschen, E., in Jochmann, G., and Hegler, C., Lehrbuch der Infektionskrankheiten, Berlin, Julius Springer, 1924, 850.
19. Sabin, A. B., *Brit. J. Exp. Path.*, 1935, **16**, 84.
20. Ch'en, W. K., *Proc. Soc. Exp. Biol. and Med.*, 1934–35, **32**, 491.
21. Li, C. P., and Rivers, T. M., *J. Exp. Med.*, 1930, **52**, 465.
22. Craigie, J., and Wishart, F. O., *J. Exp. Med.*, 1936, **64**, 803.

EXPLANATION OF PLATE 8

Fig. 1. Photograph of a preparation of elementary bodies in a relatively pure state obtained from chorio-allantoic membranes of chick embryos infected with vaccine virus. Morosow stain. × 1200.



1

Photographed by Joseph B. Haulenbeek

(Smadel and Wall: Elementary bodies of vaccinia)

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PLASMA CHOLESTEROL SATURATION IN PATIENTS WITH HYPERTENSION. WITH A NOTE ON PREPARATION OF GLASS FILTERS FOR MICRO-FILTRATION OF CHOLESTEROL DIGITONIDE

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Various authors during the past 25 years have attributed to hypercholesterolemia a rôle in initiating the arterial lesions of essential hypertension. The basis of this theory has been disproved by several recent papers (1, 2, 3), which have shown that plasma cholesterol, both free and esterified, is entirely normal in essential hypertension. However, Alvarez and Neuschlosz (4) have presented a series of experiments which indicated that the serum of patients with arterial hypertension may be supersaturated with cholesterol, even though the concentration does not exceed normal limits. Medvei (5) was unable to confirm Alvarez and Neuschlosz, and the disagreement in experimental results has remained unsettled. We have accordingly performed a series of saturation experiments on plasma from subjects with and without hypertension.

EXPERIMENTAL

Plasma was used in preference to serum because it approximates closer to the true circulating fluid. Heparin was chosen as anticoagulant in order to minimize changes in cell-plasma equilibria which might conceivably affect the state of plasma cholesterol; also to avoid the saponification of cholesterol esters by oxalate or citrate, pointed out by Shope (6). The experiments were carried out at 37°C. in a further effort to approximate *in vitro* conditions.

To obtain plasma for each saturation experiment, 20 cc. of blood obtained by venipuncture were run into a 50 cc. pyrex flask containing about 10 mgm. of heparin. The blood was whirled in the flask to

TABLE I

Plasma Cholesterol Values before and after Saturation with Free Cholesterol

Patient	Hospital number	Age	Diagnosis	Blood pressure	Plasma cholesterol				Total lipid carbon	Free cholesterol
					Free		Total			Degree of saturation
					Before saturation	After saturation	Before saturation	After saturation		
Control Group										
		years		*mm. Hg*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*mgm. per cent*	*per cent*
R. H.		25	Healthy	122/82	49	53	208	202		
B. M.		31	Healthy		62	66	220	233		94
G. Z.	9696	49	Pneumonia—convalescent	118/88	56	57	176	173		98
H. Y.	9693	40	Pneumonia—convalescent	118/80	59	59	194	196		100
M. S.	9723	48	Pneumonia—convalescent	110/70	50	50	196	203		100
Hypertensive Group										
L. S.	9490	53	Benign hypertension	184/112	45	41	158	162		110
W. A.	9399	35	Benign hypertension	117/112			269	266		
R. J.	9452	26	Malignant hypertension	174/118	79	80	251	259		99
A. R.	9506	46	Malignant hypertension	260/160	56	54	175	169		104
T. G.	9543	37	Malignant hypertension	210/122	60	64	200	209		94
P. M.	9480	24	Malignant hypertension	174/108	48	45	153	149		107
J. C.	9839	40	Malignant hypertension	269/146	90	90	289	291	742	100
Nephritic Group										
L. C.	9465	24	Chronic hemorrhagic Bright's disease	118/86	58	53	186	186		109
F. B.	9525	30	Chronic hemorrhagic Bright's disease	186/108	110	111	264	270		99
	(4 hours after 100 cc. of olive oil by mouth)				134	132	306	320		101
A. C.	8658	22	Chronic hemorrhagic Bright's disease	240/116	42	42	133	145	396	100
G. P.	8740	22	Chronic hemorrhagic Bright's disease	164/100	101	100	355	354	765	101
A. C.	9266	30	Chronic hemorrhagic Bright's disease	124/74	152	154	432	433	1110	99

assure complete mixing of the heparin, poured into a heavy centrifuge tube, and centrifuged at 2500 r.p.m. for 45 minutes. After pipetting off the supernatant plasma, it was divided into two equal portions of about 5 cc. each in 30 cc. pyrex flasks. About 50 mgm. of cholesterol were added to the contents of one flask and thoroughly suspended by gentle shaking. Both flasks were then closed with rubber stoppers and placed in an incubator at 37°C. for 6 hours.[1] At the end of this period, the sample of plasma without added cholesterol was filtered with gentle suction through a 4 cm. Buchner funnel fitted with a close-fibered filter paper. The sample with added cholesterol was then filtered through the same paper, with care to discard the first 1 to 2 cc. of filtrate. Both filtrations were carried out in the warm room at 37°C. Free and total cholesterol determinations were made in duplicate on each sample by the method of Kirk, Page, and Van Slyke (7), with minor modifications. In a few cases, total lipid carbon was determined by the method of the same authors. The results are shown in Table I.

DISCUSSION

It is apparent from Table I that no pronounced changes in cholesterol content of the plasma samples studied were brought about by saturation with added cholesterol. Both free and total cholesterol remained essentially the same. Such variations as did occur are not consistent in direction and therefore are probably without real significance.

SUMMARY

No evidence of a relationship between blood pressure and plasma cholesterol saturation was found in a series of cases which included individuals with malignant hypertension, benign hypertension, and chronic hemorrhagic nephritis. The plasma was approximately saturated with regard to free cholesterol in all cases.

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[1] Incubation for longer periods has no effect on the results obtained and, unless sterile precautions are taken throughout, is certain to result in contamination by bacterial growth. Mechanical agitation during the saturation period had no effect on the cholesterol content of plasma samples.

Note on Preparation of Sintered Glass Filters for Micro-Filtration of Cholesterol Digitonide

Kirk, Page and Van Slyke (7) used filter sticks with detachable tips for filtration and washing of the cholesterol digitonide precipitate. The filtering disk was of porous alundum. As the original supply of alundum in the laboratory was exhausted, it proved difficult to obtain more which would resist the chromic acid combustion fluid for many analyses. We have accordingly changed the porous disk from alundum to sintered pyrex glass. The filter sticks can be prepared with the sintered glass disks as follows.

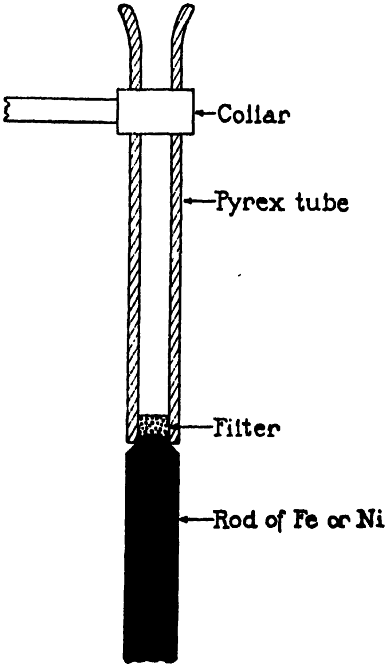

FIG. 1. Method for sintering glass filters.

Bits of broken pyrex laboratory ware are ground to a powder in a large mortar. This powder is shaken on a 100-mesh screen and the screenings reserved, while the tailings are reground and rescreened until a sufficient stock of material is obtained. The screened powder is agitated with distilled water in a small beaker and the supernatant water carrying the finest particles is decanted after a 10-second period, the process being repeated three or four times. The residual powder is then washed on to a filter paper and washed once with a saturated solution of sodium borate (borax). The paper and its contents are then dried over a steam bath. After breaking up the resultant cake into a powder again, it is ready for use.

The detachable lower end of a Kirk-Page-Van Slyke filter stick of pyrex glass is placed upright on a short iron or nickel rod as shown in Figure 1. Tubing of about 6 mm. internal diameter and 2 mm. wall thickness in lengths of about 8 cm. is used. The end of the metal rod is beveled at an angle of 45°. The bevel results in the formation of a shoulder on the finished disk, a feature of some importance.

An amount of the powdered pyrex glass prepared as above is run into the upper end of the tube, so that the upper level is somewhat above that indicated in the diagram. The matrix is then packed and smoothed by gentle tamping with a blunt glass rod. After tamping the matrix, it should have a thickness of at least 3 mm.

An air-gas blast is adjusted to deliver maximum heat intensity and brought to bear on the lower end of the tube and upper end of the metal rod, while slowly rotating about them. The upper end of the rod should reach a white heat, to insure a uniform face on the filter. The matrix will sinter at a bright red heat. The exact conditions of heating can be determined with a few trials. If the matrix is insufficiently heated the particles in the center will not adhere to each other, while excessive heating will fuse the matrix into a solid mass. With a little practice, both of these exigencies may be avoided. The method is rapid, as the three operations of sintering a filter disk, fusing it into tubing and fire-polishing the end of the tubing are performed at one time. It is not adaptable for making filters larger than 1 cm. in diameter.

BIBLIOGRAPHY

1. Page, I. H., Kirk, E., and Van Slyke, D. D., Plasma lipids in essential hypertension. J. Clin. Invest., 1936, **15**, 109.
2. Landé, K. E., and Sperry, W. M., Human atherosclerosis in relation to the cholesterol content of the blood serum. Arch. Path., 1936, **22**, 301.
3. Elliot, A. H., and Nuzum, F. R., Cholesterol content of whole blood in patients with arterial hypertension. Arch. Int. Med., 1936, **57**, 63.
4. Alvarez, C., and Neuschlosz, S. M., Untersuchungen über das Blutcholesterin bei arteriellem Hochdruck. Klin. Wchnschr., 1931, **10**, 244.
5. Medvei, C. V., Zur Frage des Blutcholesterins bei arteriellem Hochdruck. Klin. Wchnschr., 1932, **11**, 414.
6. Shope, R. E., Differences in serum and plasma content of cholesterol ester. J. Biol. Chem., 1928, **80**, 125.
7. Kirk, E., Page, I. H., and Van Slyke, D. D., Gasometric microdetermination of lipids in plasma, blood cells, and tissues. J. Biol. Chem., 1934, **106**, 203.

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MICRODETERMINATION OF CHLORIDE IN BIOLOGICAL FLUIDS, WITH SOLID SILVER IODATE

I. GASOMETRIC ANALYSIS*

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(Received for publication, May 8, 1937)

When a chloride solution is shaken with silver iodate powder, the reaction which takes place is

$$\text{(1)} \qquad \underset{\text{soluble}}{NaCl} + \underset{\text{insoluble}}{AgIO_3} \rightleftarrows \underset{\text{insoluble}}{AgCl} + \underset{\text{soluble}}{NaIO_3}$$

Although both AgCl and $AgIO_3$ are "insoluble" salts, the AgCl is so much the more insoluble that, under the conditions of this method, the reaction is almost quantitative from left to right; and it is completed within a minute. The iodate which goes into solution by the reaction serves as an accurate measure of the chloride present.[1]

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* A preliminary report of the work of this and Papers II and III (Sendroy, 1937, *a*, *b*) was given at Detroit, April, 1935, at the Meeting of the Federation of American Societies for Experimental Biology. This report (Sendroy, 1935) was apparently overlooked by Haslewood and King (1936) who have since its appearance published a paper entitled "A new iodometric procedure for the estimation of chloride in small amounts of blood." Their procedure is based, like the author's, on the use of silver iodate as a chloride precipitant, but their application is limited to Somogyi filtrates (1930) of serum and to titrimetric estimation of the iodate. The details of technique are quite different from those developed and preferred by the writer.

[1] Other "insoluble" silver salts, also more soluble than silver chloride, could be used in place of the iodate. Thus two, which the author has used with some degree of success before abandoning them in favor of the present procedure, are the chromate and the oxalate, for the reactions:

$$(a) \qquad 2NaCl + Ag_2CrO_4 \rightleftarrows \downarrow 2AgCl + Na_2CrO_4$$

This reaction offers several advantages for chloride analyses. The determination of the dissolved iodate affords a *direct* measure of the chloride, whereas measurements by difference, such as the Volhard titration used in most biological analyses, require two standard solutions and involve the cumulative errors of their measurements. These errors may be large when the concentration of chloride is relatively low. Furthermore, iodate is beautifully adapted to microanalyses, gasometric, titrimetric, or colorimetric. It is particularly suitable for the measurement of minute amounts of chloride, because in the oxidation-reduction reactions by which the iodate is measured, it functions as a hexavalent oxidizing agent. Thus 1 equivalent of chloride yields 6 equivalents of iodate to measure. These 6 equivalents are measured gasometrically by reaction with hydrazine as 1.5 moles of N_2; they are measured titrimetrically or colorimetrically by reaction with KI as 6 equivalents of free iodine. By the gasometric or titrimetric methods a precision of ±0.5 per cent can be obtained with as little as 0.1 cc. of serum. The colorimetric measurement is less exact.

The silver iodate procedure is applicable to salt solutions, to urine, to protein-free filtrates of plasma and of whole blood, and to plasma and serum without removal of proteins.

Of the three types of procedure for measuring the dissolved iodate, the writer prefers the gasometric, because of its speed, convenience, and freedom from the use of standard solutions. Fourteen or fifteen consecutive iodate determinations can be made gasometrically in an hour, with duplicates seldom differing by more than 1 part in 200.

In this paper the theoretical and experimental basis of the

(*b*) $$2NaCl + Ag_2C_2O_4 \rightleftarrows \downarrow 2AgCl + Na_2C_2O_4$$

The dissolved chromate or oxalate can be measured either gasometrically or titrimetrically. The chromate can also be measured colorimetrically, as in Isaac's (1922) colorimetric chloride method. For present purposes iodate, however, proved to be preferable. In what follows, it will be convenient to regard total halide (chloride, bromide, and iodide) simply as "chloride," since chloride is the predominant halide, especially in most biological material. However, under the conditions prescribed, $AgIO_3$ may be used for the determination of bromides and, in some cases, of iodides, as well as of chlorides.

reaction of chloride with silver iodate will be developed, and the gasometric method will be described in its macro, micro, and ultramicro forms. The titrimetric and colorimetric methods will be detailed in the accompanying papers.

In the gasometric method the iodate is measured in the Van Slyke-Neill (1924) manometric apparatus by the N_2 evolved instantaneously, according to the equation:

$$2NaIO_3 + 3N_2H_4 = \uparrow 3N_2 + 2NaI + 6H_2O \tag{2}$$

This reaction was introduced by Riegler (1902) for gasometric measurement of iodate, although not in connection with chloride analysis. The reaction was applied by Van Slyke, Hiller, and Berthelsen (1927), with the Van Slyke-Neill apparatus, to measure the iodate obtained in their gasometric methods for sulfate and total base.

The only gasometric chloride method previously described is that of Riegler (1901). He precipitated AgCl and caused the isolated precipitate to react with hydrazine in alkaline solution.

$$(c)\quad 4AgCl + N_2H_4 + 4NaOH \rightarrow \downarrow 4Ag + \uparrow N_2 + 4NaCl + 4H_2O$$

For each mole of Cl 0.25 mole of N_2 was evolved. The present procedure yields 6 times as much N_2 and appears otherwise more practicable in detail than Riegler's, which did not attain application to biological material.

DESCRIPTION OF METHOD

Reagents—

Approximately 0.85 M *Phosphoric Acid Solution*—5.8 cc. of syrupy orthophosphoric acid, H_3PO_4 (specific gravity 1.7), of reagent grade are diluted to 100 cc. with water. *This solution must be tested for and found free of halides.*

Approximately 0.34 M*, 0.17* M*, and 0.085* M *Phosphoric Acid Solutions*—0.85 M solution is diluted 2.5, 5, and 10 times, respectively, with water.

M*/15 Potassium Acid Phosphate Solution*—9.08 gm. of KH_2PO_4, diluted to 1 liter with water.

Caprylic Alcohol—This is used to prevent foaming in biological samples.

Silver Iodate, Powder, C.P.[1]—This should be kept in an amber, glass-stoppered bottle and stored in a desiccator when not in use. Under these conditions, it keeps indefinitely.

Alkaline Hydrazine Solution—Equal volumes of saturated aqueous solution of hydrazine sulfate (Merck) and of 40 per cent (40 gm. per 100 cc. of solution) sodium hydroxide (Merck's Reagent) are mixed. *Before use, the freshly prepared alkaline hydrazine solution must be first cooled to room temperature, then saturated with air.* This may be accomplished by vigorous shaking with air for 30 minutes, or by allowing it to stand overnight in a loosely stoppered flask. Some alkaline hydrazine solutions used in this laboratory have shown marked signs of deterioration over periods of several weeks, yielding low results for iodate. As a general measure of precaution *no alkali-hydrazine mixture more than 21 days old should be used for gasometric iodate determinations.*

Tungstic acid reagent (*Folin and Wu, 1919*) is prepared as modified by Van Slyke and Hawkins (1928). 1 volume of 10 per cent sodium tungstate is mixed with 8 volumes of N/12 sulfuric acid. The solution must be freshly prepared every 2 weeks.

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[1] The silver iodate can be prepared as follows from two solutions: (1) *potassium iodate* (Merck's Reagent), 23 gm. in 600 cc. of water, and (2) *silver nitrate* (Merck's Reagent), 18 gm. in 400 cc. of water. To the iodate in a 2 or 3 liter beaker, the silver is added from a burette or dropping funnel, slowly, with constant stirring. After standing overnight in the dark, the precipitate is poured onto a Buchner funnel, and washed three times by decantation. It is then washed repeatedly on the filter with 100 cc. portions of water, to free it of excess iodate. Near the end of this washing process (about twelve washings) the 100 cc. portions of wash water are collected after each washing, and a 10 cc. sample taken into a test-tube. A few drops of 0.85 M H_3PO_4 and a few drops of 5 per cent potassium iodide are added to develop the yellow iodine color. Washing is continued until the yellow color becomes constant. The material is dried *in vacuo* at room temperature, overnight. It is then pulverized and stored in an amber, glass-stoppered bottle, and kept in a desiccator when not in use. The theoretical yield is about 30 gm. We have actually obtained 27 gm. of dried material, enough for from 270 to 4500 determinations. The purity of the material may be further tested (1) by a determination of its solubility in water, and (2) by its reaction with known chloride solutions. These determinations are discussed in the experimental sections. During these studies, silver iodate was not available on the market. Since then, Merck and Company, Inc., have prepared some according to the above directions. Their material, tested under a variety of conditions, has been found to be the same as that prepared by the writer.

Zinc hydroxide reagent (*Somogyi, 1930*), prepared as described by Peters and Van Slyke (1932).

Solution I—10 per cent zinc sulfate. 100 gm. of $ZnSO_4 \cdot 7H_2O$ in 1 liter of solution.

Solution II—0.5 N sodium hydroxide.

The two solutions must be so related that when 10 cc. of the zinc sulfate are titrated with sodium hydroxide, 10.8 to 11.2 cc. of the alkali are required to produce a permanent pink color with phenophthalein. The 10 cc. of solution are diluted with 50 to 70 cc. of water before the titration; the alkali is added slowly with continuous shaking.

Procedure

The general procedure consists of the following steps.

1. The sample is diluted in an acid solution (usually with 0.085 M phosphoric acid) to a pH of between 2.0 and 3.0, and to a chloride concentration of between 12 and 3 mM per liter.

2. With the addition of solid silver iodate and vigorous shaking, the chloride is precipitated and iodate is released into solution.

3. The silver chloride, together with excess silver iodate, is separated from the solution by centrifugation at high speed.

4. The supernatant liquid is then analyzed gasometrically for iodate.

5. A *blank* analysis, which serves for a whole series of gasometric measurements, is carried out.

The details of the steps involved are as follows:

1. Preliminary Preparation and Dilution of Sample for Analysis—The purpose of this treatment is to approximate, in the solution to be analyzed, concentrations of chloride and of hydrogen ions which have experimentally been found suitable for the chloride precipitation reaction. Dilution serves not only to facilitate the analysis by avoiding unnecessarily strong and impracticable concentrations of iodate, but also to decrease the concentrations of proteins and other possibly interfering substances, when present. The preliminary treatment and preparation will therefore vary somewhat with the nature and the available amounts of the material to be analyzed.

In general, a sample of any amount of original material, down to 0.02 cc., may be initially used. Depending on its chloride concentration, it is diluted with 0.085 M or 0.17 M phosphoric acid solution to a chloride concentration of between 3 and 12 mM. In any case, regardless of how much or how little dilution may be necessary to bring about these concentrations, as a rule (exceptions to which will be indicated further) the pH of the dilute solution should be brought to about 2.0 to 3.0 with phosphoric acid. The procedure for diluting various biological materials will be described in connection with their analyses.

Phosphoric acid has been adopted for general use in this method, because it has been found to answer the requirements of the proper conditions for serum analysis. In the analysis of urine samples slightly high results were sometimes obtained when the pH was over 3.5 or 4.0. Similarly, when M/15 KH_2PO_4, of pH 4.5, was used to dilute plasma or serum 10-fold, the average results were 2 per cent higher than when the chloride was determined by digestion methods. Furthermore, when serum was diluted more than 1:10 with M/15 KH_2PO_4, even before $AgIO_3$ was added, a precipitate appeared in the sample; probably serum albumin, the isoelectric point of which is approximately at pH 4.7. For both urine and serum a lower pH proved more satisfactory for the reaction of chloride with $AgIO_3$. Although acetic acid also had been used for urine, it was unsatisfactory for serum. A suitable acid, not reacting with silver, iodate, chloride, or protein, was found in orthophosphoric acid, H_3PO_4. It has therefore been used as a diluent for the procedures described in these papers.

In the development of the method we have usually carried out duplicates by precipitation of the chloride in two separate tubes, with one iodate analysis of the supernatant fluid from each. Very often each supernatant fluid was analyzed in duplicate. A significant deviation in the duplicate precipitation results has been so rare in our experience, that for *macro-* and *micro*procedures in the analysis of any of the materials mentioned we recommend the precipitation of but one sample, with duplicate analyses of the supernatant fluid.

2. Precipitation of Chloride with Silver Iodate—To the dilute, acidified solution silver iodate is added, about 10 mg. per cc. of solution being required. More does no harm; less is to be avoided. With little practise it is easy to estimate the necessary amount on the tip of a spatula. The tube is then covered by a rubber cap and shaken *vigorously* for 2 minutes by hand or in a shaking machine. We have utilized the manometric apparatus itself (Fig. 1) for this purpose by wrapping a towel or cloth around the jacket of the extraction chamber and fastening the tubes together with intertwining rubber bands. The tubes should all be with their axes in a horizontal position and pointing in the same direction as the driving shaft which shakes the chamber. The reaction with silver iodate is practically instantaneous but shaking is continued to saturate the solution exactly with air, which subsequently is liberated in the gasometric determination, together with the N_2 freed by action of the iodate on N_2H_4. To make sure that the air included shall be the same as in the blank analysis, it is important to have the solutions at room temperature before the analyses are begun.

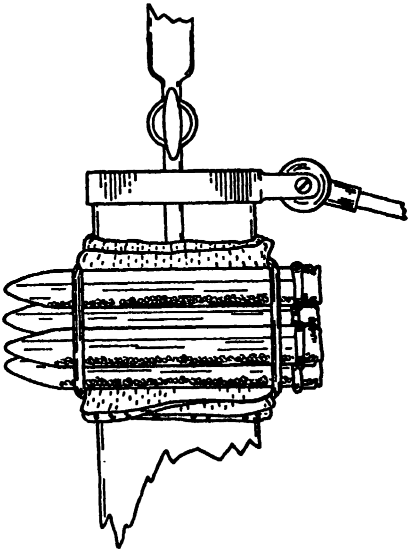

FIG. 1. Centrifuge tubes attached to outer jacket of the extraction chamber, for shaking with solid $AgIO_3$.

3. Separation of Solid Silver Salts from the Supernatant Fluid—After the shaking, subsequent sampling may be facilitated, if the caps are removed and the necks of the tubes are wiped free of precipitate. For small volumes, as in the *ultramicro* serum analysis, this is unnecessary. The tubes are covered again with clean caps and centrifuged for 1 minute[3] at a speed greater than 3000 R.P.M. The resulting supernatant liquid, which is almost completely clear, is then analyzed for iodate.

4. Analysis of Liberated Iodate in the Supernatant Fluid—Interfering substances in some materials may reduce acid solutions of iodate at an appreciable rate. Such is the case in the analysis of certain types of urines. For such material the iodate analysis should be made within 1 hour after the centrifugation. Serum samples may stand for at least 3 hours before analysis. Salt solutions and protein-free filtrates may stand for more than 24 hours without change. The number of samples simultaneously shaken with silver iodate, therefore, depends on the length of time which may be allowed to elapse between the first and last iodate determinations of the several supernatant samples.

When samples are being taken for analysis from the centrifuged tubes, care should be exercised in avoiding their contamination with solid silver iodate or chloride, from either the surface or the bottom of the centrifuged liquid. Usually it will suffice to close the stop-cock of the pipette, and then, without touching the sides of the tube, to plunge the tip of the pipette quickly below the surface. If the tip is held no nearer than ½ inch from the precipitate, the sample may be withdrawn, without contamination, by gentle suction controlled by the pipette stop-cock.

However, contamination may easily be avoided with certainty by a cotton filter device similar to that of Van Slyke and Hawkins (1928). A little cotton is twisted to a point, then sucked by vacuum into the tip of the sampling pipette. The protruding cotton is cut off at about 2 mm. from the tip of the pipette which is then plunged below the surface for withdrawal of the sample.

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[3] For salt solutions, normal urines, and protein-free filtrates, filtration through halide-free filter paper ("ashless" filter paper washed free of halide in distilled water and then dried) may be carried out to separate the silver salts from the supernatant liquid. When protein is present, however, highest accuracy is obtained only by centrifugation. Filtration of protein-containing material (serum or urine) gives less consistent and higher results (by about 1 per cent) for both the gasometric and titrimetric (Sendroy, 1937, *a*) measurements. Moreover, loss of fluid on the filter paper eliminates filtration as a practical procedure in the analysis of small volumes of supernatant fluid.

Before accurate adjustment to the upper mark of the pipette the cotton is removed. In handling small volumes of supernatant fluid, as in the *ultramicro* serum analysis, this cotton plug is a *necessary* device. In these cases the need of obtaining most of the supernatant fluid for analysis makes it necessary to hold the tip of the sample pipette so close to the centrifuged solid material that contamination would be inevitable without the protection of the cotton filter.

Gasometric Determination of Total Dissolved Iodate—As found by Van Slyke, Hiller, and Berthelsen (1927), the reaction between hydrazine and iodate is quantitative. The author has confirmed their results, but has obtained consistently accurate results only when the indicated precautions for the use of the hydrazine reagent (see "Reagents") and for the technique of iodate measurement given below were observed. Under these conditions the nitrogen yields were 100.0 ± 0.3 per cent of theoretical for dozens of known potassium iodate and biiodate analyses covering a wide range of concentrations. The results were the same whether the iodate was dissolved in water or in 0.085 M H_3PO_4 solution.

The manometric apparatus (Van Slyke and Neill, 1924; Van Slyke, 1927) must be kept scrupulously clean, and, if possible, should be reserved only for iodate determinations. If used for analyses involving other substances which may react with hydrazine or iodate, suitable precautions should be taken against contamination from such sources (*e.g.* see p. 413, Peters and Van Slyke (1932)). It is well to change most of the mercury in the system once a month, simply by evacuating the chamber as for extractions, closing the stop-cock leading to the leveling bulb, and pouring off as much of the mercury from the leveling bulb as is possible without allowing air into the rubber tubing. Fresh mercury is then used to refill the leveling bulb.

Before the first analysis of a series, the apparatus is freed of air and of any substance which might oxidize hydrazine to N_2 gas. This is done by mixing 2 cc. of the alkaline hydrazine reagent with 2 cc. of distilled water in the extraction chamber, evacuating the chamber, and shaking for 1½ minutes. The evolved gas and solution are then expelled. 10 or 15 seconds are allowed for solution adherent to the inner wall of the chamber to rise to the top above the mercury. This solution is then also expelled.

The analysis is begun by placing about 3 cc. of the alkaline hydrazine solution in the cup of the chamber. With the leveling bulb on a level with the bottom of the chamber, and its control stop-cock open, hydrazine solution is admitted into the chamber until it *exactly* reaches the 2.0 cc. mark. About 1 cc. of mercury is poured into the cup, a little is allowed to run into the chamber, and the excess hydrazine above the mercury is removed with two washings of water. Samples of the centrifuged supernatant fluid are admitted into the chamber through the mercury seal, covered with a layer of water, as described by Van Slyke (1927). (See also Peters and Van Slyke (1932) Fig. 52, p. 344.) A 1 or 2 cc. stop-cock pipette is used.[4] The leveling bulb of the apparatus is kept at the same level as for the admission of the hydrazine, and the admission of the sample is controlled by the *stop-cock at the top of the extraction chamber.* The reaction between iodate and hydrazine is so rapid that some of the evolved N_2 gas would escape up through the pipette were the sample not delivered at slight negative pressure. The pipette is slowly lifted up out of the cup, and almost all of the mercury in the cup is allowed to enter the chamber to form a seal through the stop-cock.[5]

The sample having been introduced, it is *first thoroughly mixed with the hydrazine reagent in the chamber*, by slightly raising and lowering the leveling bulb of the apparatus, and by shaking the chamber several times by hand. The chamber is then evacuated so that the mercury surface lies near the 50 cc. volume mark, about 1 cm. below the curve of the bulb. After vigorous shaking for 1½ minutes,[6] the pressure of the gas evolved is read at the 0.5 or 2.0 cc. volume, and is p_1 in the calculation. The temperature is recorded.

5. The Blank—A gasometric analysis on an equal volume of either another aliquot portion of the diluted sample or of the diluent alone is carried out simultaneously with that on the sample through steps (1), (2), (3), and (4) of the procedure outlined, exactly as above, *except that no silver iodate is used.* The result of the blank determination gives the base-line reading p_0 for all analyses, and serves to correct for the amount of air dissolved in the hydrazine reagent and in the supernatant sample analyzed. In running a series of determinations, it is well to make one gasometric measurement on a portion of the blank solution once in the beginning and another at the end of the series.

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[4] For the comparatively rare instances when less than 1 cc. of supernatant fluid is available for the gas analysis, see the detailed directions for the *ultramicro*analysis of serum, given in the following (p. 359).

[5] The error involved in the transfer of samples through a mercury seal is discussed in the section on "Calculations."

[6] Upon being first shaken, some mixtures containing biological material foam in spite of the caprylic alcohol. In such cases, the shaking should be stopped and the foam allowed to subside. Upon resumption of the shaking, no further foaming will occur.

In most of the analyses to be described, the original sample material is diluted to such an extent that it has practically no effect on the amount of air contained in the supernatant fluid used for analysis. It is therefore not necessary to use any of the original sample material for a blank analysis. Instead, the blank determination may be made on the diluent used for preparing the original material for analysis. Thus, in the analysis of serum without deproteinization, or of highly diluted urine, the blanks are made on the 0.085 M or 0.17 M H_3PO_4 solutions used for dilution. In the analysis of protein-free filtrates, the blanks are made on a mixture of the same reagents used to precipitate the proteins in the original sample, with water used in place of the serum or whole blood. *In all cases the same solution or solutions must be used for the blank that are used for the dilution of the original sample,* in order that the air content correction may truly represent that of the diluted supernatant sample analyzed for iodate. The volumes of the solutions shaken in the centrifuge tubes should be approximately the same for both the blank and the supernatant fluid analyzed. Also, the temperature and length of time of shaking, with and without $AgIO_3$, for both sample and blank, respectively, must be the same, otherwise the blank may not provide an accurate correction.[7]

Since the apparatus is automatically rendered air-free with each analysis, *no washing of the apparatus is required between analyses* of either samples or blanks. Analyses of the centrifuged iodate solutions can, therefore, be made in rapid succession, at the rate of fourteen or fifteen an hour.

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[7] The author has investigated experimentally, the possibility of eliminating the actual determination of gasometric blank readings altogether, first, by *calculating* the air content of the samples from their temperature and the barometric reading, and secondly, by making the sample air-free before analysis. It was found that the actual determination of p_0 from the blank, as described in the text, was simpler, less time-consuming, and more accurate.

Application of the above general procedure to different types of material will now be detailed.

A. Determination of Chloride in Inorganic Material—Samples are diluted with a suitable phosphoric acid solution to a final chloride concentration of 3 to 12 mM per liter, and a pH of about 2.0 to 3.0. The phosphoric acid concentration of the diluted solution should be no more than 0.17 M. Thus, approximately 0.1 M neutral salt solutions, or 0.1 N HCl solutions, would be diluted 10 to 30 times with 0.085 M H_3PO_4, then analyzed exactly as described for serum analyses below, except that no caprylic alcohol is necessary.

Solutions containing too much free acid or alkali to give the above pH when diluted with 0.085 M or 0.17 M H_3PO_4 should first be neutralized to alizarin red with sodium hydroxide (*chloride-free*) or phosphoric acid. The neutralized material is then diluted further by the addition of 0.085 M phosphoric acid. For solutions containing iodide the final pH should be somewhat higher (4.5), and dilution should be made with M/15 potassium acid phosphate. *Solutions used for dilution should be chloride-free when tested with silver nitrate and nitric acid.*

B. Determination of Chloride in Urine—The presence of albumin or blood in the urine does not prevent the application of the gasometric chloride method to the analysis of such material. A slight effect of the albumin is corrected for in the calculations. All samples (preferably obtained without preservative)[8] are diluted with 0.17 M H_3PO_4, which strength of the acid is sufficient to bring almost any sample of urine, in the dilutions employed, to a pH of 3.5 or lower. The method of diluting samples will depend on whether speed and convenience, or whether accuracy is the primary consideration.

In the following, two procedures for urine analysis will be described: (*a*) a routine rapid method applicable to all samples, and

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[8] If the use of a preservative should be necessary, it is recommended that a few crystals of thymol (Peters and Van Slyke, 1932) be used. Toluene should be avoided, as its use in any gasometric method is very likely to cause difficulties on account of its vapor tension and its air solubility.

(*b*) a more refined technique of individual treatment of samples, for maximum accuracy.

Routine Urine Method—A 0.5 cc. sample of urine is pipetted into a graduated 15 cc. conical Pyrex centrifuge tube. A drop of caprylic alcohol is added, then 0.17 M H_3PO_4 to the 10 cc. volume mark. About 0.1 gm. of $AgIO_3$ is added to the tube, which is then capped, shaken for 2 minutes, centrifuged,[9] and analyzed as described in the general procedure (steps (2), (3), and (4) above).

Samples of 1 cc. or 2 cc. of the supernatant fluid are withdrawn and analyzed gasometrically within 1 hour after centrifugation. The pressure readings are made at the 0.5 cc. volume when p_1 is not more than about 560 mm.; otherwise, when more N_2 is present, they are read at the 2.0 cc. volume. The *blank* analysis for the p_0 reading is carried out with the *same* 0.17 M H_3PO_4 solution used to dilute the urine. A 10 cc. portion is shaken at the same time, without the addition of $AgIO_3$. Samples of 1 cc. or 2 cc. are analyzed gasometrically as above.

With this technique the accuracy of the analyses of urine samples of chloride concentration between 60 mM and 250 mM per liter (3.5 to 14.6 gm. of NaCl per liter) is limited only by the accuracy with which one may dilute to volume in a graduated centrifuge tube. The tubes used for this purpose should have the 10 cc. volume mark checked by delivery of water from an accurate 10 cc. pipette. When the agreement is within 0.1 cc., an accuracy of 1 per cent may be expected of these analyses.

In the case of the relatively few urine samples of more than 250 mM of chloride per liter, the required excess of $AgIO_3$ may not be sufficient, and greater dilution than 20 times may, therefore, be necessary. In the case of samples below 60 mM in chloride, the 20-fold dilution gives supernatant samples of less than 3 mM. Under these conditions, the possible errors are increased, but an accuracy of 5 per cent or better may be obtained, which is sufficient for most clinical purposes. The errors are minimized by a correction in the calculations for the solubility of $AgIO_3$ in the supernatant samples analyzed, and by the use of 2 cc. samples of supernatant fluid with the nitrogen measured at the 0.5 cc. volume, so that maximum pressure readings may be obtained.

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[9] See foot-note 3. Most samples of urine may be filtered if centrifugation happens to be inconvenient. However, when protein is present, centrifugation is necessary to avoid errors which are further increased, if at the same time the chloride concentration is low.

In some pathological conditions the application of the *routine method* to urines with low chloride content may result in errors greater than 5 per cent. Such cases are discussed in a following paper (Sendroy, 1937, *c*). Under those conditions, and in any case in which an accuracy of better than 5 per cent must be obtained, the following *precise method should be used.*

Precise Urine Method—When it is desirable that the maximum accuracy (±0.5 per cent) be obtained for any given urine sample, the dilution with 0.17 M H_3PO_4 is so made that the diluted sample may be between 3 mM and 12 mM, preferably about 8 mM, in chloride concentration. The rest of the determination is then carried out exactly as described above. Samples of 2 cc. of the supernatant fluid are analyzed, and pressure readings are made at the 2.0 cc. volume.

Under these conditions, the extent of dilution will vary from one sample to another, depending on its chloride concentration. A preliminary analysis by the 20-fold dilution method of the above *routine procedure* may be made to determine this probable concentration, and hence the optimum dilution. There is, however, an easier procedure. An approximate indicator of the necessary dilution of any given urine sample is furnished by the reading of the specific gravity (to two decimal places, uncorrected) which, from Sendroy, Seelig, and Van Slyke's results (1934) and from our own, varies with the total base and chloride in an approximately linear manner. Chloride-poor urines obtained from patients on low salt diets also have a linear relationship, but one which is quantitatively different from that of normal urines. Such samples require relatively less dilution. Thus, the dilution for any sample will be determined by its specific gravity, as indicated in Table I. Samples of 1 cc. are diluted in volumetric flasks to the final volume indicated.[10] For dilution to 3 times the volume, 5 cc. of urine are diluted in a 15 cc. flask, or 2 volumes of 0.17 M H_3PO_4 are added to 1 volume of urine. By these dilutions the chloride will be within the desirable limits of concentration of 3 and 12 mM in all but a very few cases.

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[10] Volumetric flasks of 5 cc., 20 cc., or 30 cc. capacity are not carried in stock as standard equipment in supply houses in this country. They may easily be obtained, on special order, from several of the more prominent glass-blowing firms. We have imported ours directly from Gebruder Schubel, Frauenwald i. Thur., or Fettke & Co., Döbern, N.-L., Germany.

The gasometric analysis of 2 cc. of the supernatant fluid is carried out as described above for the *routine procedure*. The *blank* analysis is likewise made on the same 0.17 M H_3PO_4 used to dilute the urine. In the rare cases in which the maximum accuracy may be required in the analysis of chloride-poor samples, if the dilution used is 1:3, the blank analysis should be made on another portion of approximately the same volume of diluted urine, treated in the same way as is the one analyzed for chloride, except that no $AgIO_3$ is used.

TABLE I

Dilutions of Urine Samples According to Specific Gravity, for Analysis by the Precise Method

Specific gravity (uncorrected) of sample of urine	Dilution with 0.17 M H_3PO_4 of 1 volume of urine to multiple of the volume indicated	
	Normal urine	Low chloride urine
1.005	5	3
1.010	10	
1.015	15	
1.020	20	5
1.025	25	
1.030	30	10

C. Determination of Chloride in Plasma or Serum, without Deproteinization—Samples, which may be from 1.00 cc. to as little as 0.02 cc., are diluted with 0.085 M phosphoric acid to from 10 to 31 times the original volume. The larger samples (1.0 and 0.5 cc.) are easier to pipette accurately. On the other hand, scarcity of material, as in pediatric work or in experiments on small animals, may make desirable the use of 0.02 to 0.2 cc. Indeed, the author has analyzed samples of 0.02 cc. gasometrically and titrimetrically with an accuracy closely approximating the results obtained with larger amounts of material (Table XXI). A nitrogen pressure of about 90 mm. is available for measurement in the gasometric analysis of 0.02 cc. samples. In what follows, for the sake of convenience, the procedure will be classified as *macro*, *micro*, or *ultramicro*, depending upon the size of the sample used, 0.5 cc., 0.1 cc., or 0.02 cc. The same procedures are applicable to non-protein-containing fluids, the chloride concentration of which approximates 0.1 M.

Macro Plasma Analysis—A sample of 0.5 cc. of serum or plasma is delivered from an Ostwald pipette, calibrated between marks, into a 10 cc. volumetric flask. 1 drop of caprylic alcohol is added. The sample is then diluted to the mark with 0.085 M phosphoric acid. As a general procedure for plasma or serum, the macroprocedure with 0.5 cc. samples will be found satisfactory from the standpoint of convenience (only one pipetting being required for the dilution) and sufficiency of supernatant sample for the iodate analysis.

The diluted sample is transferred to a 15 cc. Pyrex centrifuge tube, 0.1 gm. of $AgIO_3$ is added from the tip of a spatula, and the tube is covered with a rubber cap. It is then shaken for 2 minutes, centrifuged for 1 minute, and analyzed gasometrically, as described in detail in the general procedure in sections (2), (3), and (4) above.

Samples of 1.0 cc. of the supernatant solution are taken into a 1 cc. stop-cock pipette usually without a cotton plug.[11] The pressure readings p_1 for the gasometric analyses are taken at the 0.5 cc. volume.

Blank—In another centrifuge tube 10 cc. of the same 0.085 M H_3PO_4 used for the dilution of the serum are shaken *without $AgIO_3$* and centrifuged simultaneously with the dilute serum + $AgIO_3$. A 1 cc. sample is used for the gasometric analysis, and the pressure reading, p_0, is taken at the 0.5 cc. volume. If a series of gasometric analyses requiring over an hour is run, duplicates of the blank are made on two 1 cc. aliquots of the same solution, one at the beginning and the other at the end of the series.

Micro Plasma Analysis—Samples of 0.2 cc. or 0.1 cc. of plasma or serum are delivered from pipettes calibrated *to contain* into 15 cc. Pyrex centrifuge tubes into which there have previously been accurately pipetted 5 or 3 cc., respectively, of 0.085 M H_3PO_4. In the former case, a *small* drop of caprylic alcohol (0.01 cc.) is added, while in the latter case, the end of a wire dipped in caprylic alcohol is immersed in the solution. For the 0.2 cc. and 0.1 cc. analyses about 50 mg. and 30 mg. of $AgIO_3$ are added, respectively. The rest of the analysis is carried out in detail exactly as for the *macro*determination. Samples of 1 cc. of the supernatant fluid are taken with a stop-cock pipette equipped with a cotton plug. The *blank* analysis is carried out as above, without $AgIO_3$, with 5 cc., or 3 cc. of the 0.085 M H_3PO_4 used for the serum dilution.

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[11] In the analysis of plasma or serum, a thin layer of fat-like substance mixed with caprylic alcohol may sometimes be present on the surface of the centrifuged solution. This should be dispersed throughout the liquid by gentle stirring before samples are withdrawn into a pipette equipped with a plug of cotton, as described in section (4) above.

Ultramicro Plasma Analysis—Samples of 0.05 cc. or 0.02 cc. are delivered from pipettes calibrated *to contain*, into small Pyrex test-tubes (13 × 100 mm.) into which there has previously been accurately pipetted 1.5 cc. or 0.6 cc., respectively, of 0.085 M H_3PO_4. A very *thin* wire dipped into caprylic alcohol and briskly shaken free of adhering fluid is touched to the surface of the solutions. For the 0.05 cc. and 0.02 cc. analyses, about 15 mg. and 6 mg. of $AgIO_3$ are added, respectively. *For the 0.05 cc. analysis*, the rest of the procedure is carried out in detail exactly as for the macrodetermination. Samples of 1 cc. of the supernatant fluid are taken with a stop-cock pipette equipped with a cotton plug. The *blank* analysis is carried out with 1.5 cc. of the same 0.085 M H_3PO_4 used for the serum dilution.

For the 0.02 cc. analysis, the procedure is similar, but with a slight difference in the gasometric analysis of the supernatant fluid. The small volume of supernatant fluid in this case (0.62 cc.) makes necessary the use of a 0.5 cc. pipette *without a stop-cock*, calibrated between marks, the lower mark being near the tip. A cotton plug filter is used in the pipette. After admission of the alkaline hydrazine to the manometric chamber the cup is washed thoroughly with water, the sample of supernatant fluid is pipetted into the cup, then likewise admitted into the extraction chamber. The lower part of the cup is washed with 1.0 cc. of water measured from a pipette, and this also is allowed to run into the chamber before the mercury seal is made. The volume of wash water should be within 0.01 cc. the same as in the blank analysis, because the dissolved air would affect the results if the variation were greater. The *blank* analysis is carried out exactly as above with 0.6 cc. of the same 0.085 M H_3PO_4 used for the serum dilution.

Measurement of Ultramicro and Micro Samples—The point in the *ultramicro-* and also in the *micro*method, where accuracy demands especial precaution, is not in the subsequent measurement of the iodate, but in the measurement of the small samples of serum or other sample material in pipettes calibrated *to contain.* The precautions add little to the time required, but the procedure is not a casual one and requires a little practise before good results are obtained. The major difficulty seems to lie not in the calibration or of the reading of the volume of the pipette, but in securing a volume of sample corresponding to that of mercury held between the tip and the volume mark when the pipette is calibrated. Discrepancies in this respect are usually the result of inadequate wiping of the tip. As a result, there may be traces of sample adhering to the outside around the tip. If the tip itself is actually touched with the wiping material, cloth, or filter paper, there may be an imperceptible loss at that point, because capillary attraction may withdraw the fluid a little from the pipette tip to the wiping material. On the other hand, if the tip is not wiped well enough, there may be an imperceptible protuberance of solution from the pipette, and an increased volume of sample. The author has adopted a simple, rapid technique for pipetting such small volumes with accuracy. Adherence to the following precautions has been found absolutely necessary.

In the first place, the pipettes used must be scrupulously clean and free flowing. They should be kept in chromic acid cleaning mixture when not in use. Before a pipette is used, it is thoroughly rinsed with distilled water, then with redistilled acetone (Commercial Solvent, or C.P. grade), and is dried in a few seconds with an air current. The sample to be analyzed is drawn up into the pipette only about 4 mm. above the calibration mark. The outside of the pipette, within several cm. of the tip, is then flushed with a thin stream of distilled water, after which the tip is slightly immersed in a small beaker containing about 50 cc. of water. The sample solution is then allowed to run down *slowly* into the water, until the meniscus reaches the mark. The tip is at once lifted from the water and is carefully wiped free of water with halide-free filter paper. The tip and the meniscus are again inspected to make certain there has been no additional drainage owing to capillary action. If the inspection is satisfactory, the pipette is immersed in the previously measured portion of phosphoric acid, and washed up and down five or six times. In the washing process the solution should not be drawn more than about 2 mm. above the pipette calibration mark.

In the *ultramicro*procedure, measurement of the sample and inspection of the tip are best made with the aid of a magnifying lens clamped to a ring-stand. The ultramicropipette is made of capillary tubing of about 0.5 mm. bore. A length of about 23 cm. and of 9 cm. from the tip corresponds to a volume of 0.05 cc. and 0.02 cc., respectively. For the calibration of micropipettes see Peters and Van Slyke ((1932) p. 18).

D. Determination of Chloride in Protein-Free Filtrates of Plasma, Serum, or of Whole Blood—Deproteinization of plasma or serum is unnecessary for the gasometric or titrimetric analysis. Techniques for use with the tungstic acid filtrate of Folin and Wu (1919) and the zinc hydroxide filtrate of Somogyi (1930) will be described, however, because, when these filtrates are prepared for other analyses, it may be convenient to use them for chloride also. *Removal of proteins from whole blood is necessary.* For blood analysis, tungstic acid filtrates are recommended.

(*a*) *Tungstic acid filtrates of plasma or serum* may be used, prepared according to Folin and Wu (1919) or according to the modification of Van Slyke and Hawkins (1928) who mixed the sulfuric acid and sodium tungstate before use. In these filtrates the plasma is diluted to 10 times volume. Caprylic alcohol is added before deproteinization.

As much of the filtrate as is available, up to 10 cc., is transferred to a 15 cc. Pyrex centrifuge tube and $AgIO_3$ is added, 10 mg. per cc. of filtrate. The rest of the analysis is carried out as described above under "Procedure" (sections (2), (3), (4), and (5)).

Samples of 1 or 2 cc. of the supernatant fluid may be taken for gasometric iodate determination with the N_2 pressure measured at 2.0 cc. volume. If there is a scarcity of filtrate, samples of 0.5 cc. of the supernatant fluid taken in a pipette without a stop-cock may be used, and the N_2 pressure measured at 0.5 cc. volume, as described above for the ultramicroanalysis without deproteinization of 0.02 cc. of serum.

A *blank* analysis is made on another portion of tungstic acid filtrate treated in every detail as the one analyzed for chloride, except that no $AgIO_3$ is used. If the amount of available filtrate is insufficient, the blank may be carried out on an approximately equal volume of a mixture of the same tungstic acid reagents, with water used in place of plasma or serum.

(*b*) *Tungstic acid filtrates of whole blood* may be used, *but not the usual Folin-Wu filtrate.* The dilution of the sample is to 25 times the volume, and a change in the addition of the reagents is required.

Macrodetermination—In a 25 cc. volumetric flask, a 1 cc. sample of blood, about 3 cc. of water, and 1 drop of caprylic alcohol are placed. There are then added about 15 cc. of the mixed Van Slyke-Hawkins reagent, slowly, with stirring. Finally, 0.34 M H_3PO_4 is added to the volume mark. The flask is vigorously shaken, allowed to stand ½ hour, and the filtrate separated from the precipitated protein[12] by centrifugation. 10 cc. of the filtrate are treated with $AgIO_3$, as in the case of serum filtrates.

Microdetermination—To a 0.2 cc. sample of blood in a 5 cc. measuring flask are added 0.5 cc. of water, about 0.01 cc. of caprylic alcohol, 3.0 cc. of tungstic acid reagent, and 0.34 M H_3PO_4 to the mark. The filtrate is separated from the precipitated protein, as above. As much of the filtrate as is available is treated with $AgIO_3$.

Analysis of these blood filtrates is carried out as described above for serum filtrates. Samples of 1.0 cc. of the supernatant fluid are analyzed, with N_2 pressure measured at the 0.5 cc. volume. Blank analyses are made on equal volumes of filtrate, or reagents, as in serum filtrate analyses.

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[12] When enough sample (1 cc. of blood) is available, filtrates may be separated from the precipitated protein by filtration through ashless filter paper previously washed free of chloride in distilled water and dried. However, more "filtrate" is secured by centrifugation, which method alone can be used in the deproteinization of small samples. For convenience we shall refer to the centrifuged solution after addition of $AgIO_3$, as "supernatant" fluid, to the protein-free solution, however, obtained after addition of a protein precipitant, as "filtrate."

(*c*) *Zinc hydroxide filtrates of plasma or serum* may be used, prepared as usual, by dilution to 10 times the volume, with the reagents of Somogyi (1930), as described by Peters and Van Slyke ((1932) p. 481). The filtrate is further prepared for analysis by the accurate addition of 1 volume of 0.85 M H_3PO_4 solution to 10 volumes of filtrate.[13] The procedure from this point on is the same as that described for the analysis of tungstic acid filtrates of serum described above. Depending upon the amount of supernatant fluid available, gasometric determinations are made with 1.0 or 2.0 cc. samples with gas measurements at 2.0 cc. volume, or with 0.5 cc. samples with gas measurements at 0.5 cc. volume. *Blank* analyses are carried out with an approximately equal volume of another portion of filtrate.

If the filtrate available should be insufficient, the blank may be carried out just as well on approximately an equal volume of the same mixture of Somogyi reagents and 0.85 M H_3PO_4 with water used in place of the serum.

Calculations

The pressure, P, of N_2 evolved by reaction of iodate and hydrazine is $P = p_1 - p_0$. p_1 is the manometer reading obtained in the iodate determination of the supernatant fluid of the chloride precipitation. p_0 is the reading observed in a blank analysis at the *same temperature* as the p_1 reading. The chloride concentration is obtained by multiplying P by the proper factor from Tables III, IV, and V.

Temperature Correction for p_0—When the temperature changes between the p_1 and p_0 readings, it is not necessary to carry out another blank determination in order to obtain the correct p_0. One merely corrects the observed p_0 to the temperature of the p_1 reading by adding or subtracting the correction indicated in Table II. The p_0 reading is a function of the air contained in the sample and reagents, and extracted in the chamber during the analysis, and of the vapor tension of the mixed solutions. At any given temperature, the vapor tension will be fixed at a definite value, but the air content will vary with the volume S of the extracted solutions. A change in temperature will cause a change in both components of the p_0 reading. The corrections to p_0 in Table II are, therefore, given as a function of temperature change and of the total volume S of the extracted reagents in three ranges of temperature.

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[13] For routine work it may be found more convenient to put 1 volume of 0.85 M H_3PO_4 solution in a volumetric flask of 10 volumes capacity, and to dilute to the mark with the filtrate.

Correction for Loss of Sample in Transfer Through a Mercury Seal—Van Slyke (1927) has called attention to the error involved in the transfer of solution from a pipette to the chamber, through a mercury seal, without washing. He found a loss of from 0.005 cc. to 0.007 cc. of sample when the pipette was withdrawn from the cup. He, accordingly, recommended that the mercury seal be used only for samples of 2 cc. or greater volume.

Table II

Temperature Corrections for p_0

When the temperature of the p_1 reading is *higher* than that of the blank p_0 reading, the correction is *added* to the observed p_0; when p_1 temperature is *below* p_0 temperature, the correction is *subtracted* from p_0. Δt is the temperature change between the p_1 and p_0 readings. S is the total volume of solution extracted during the analysis, and includes 2 cc. of alkaline hydrazine solution in each case, the sample, and wash water when used. a is the volume mark of the extraction chamber at which p_1 and p_0 are read.

Δt..............		0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0	Range of temperature readings
S	a	Corrections to p_0										
cc.	*cc.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	°*C.*
3-6	2.0	0.1	0.2	0.2	0.3	0.4	0.5	0.6	0.6	0.7	0.8	16-20
3-4	0.5	0.1	0.2	0.3	0.4	0.5	0.5	0.6	0.7	0.8	0.9	
4.5-6	0.5	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	1.0	1.1	
3-6	2.0	0.1	0.2	0.4	0.5	0.6	0.8	0.9	1.0	1.2	1.3	21-25
3-4	0.5	0.1	0.3	0.4	0.6	0.7	0.8	1.0	1.1	1.2	1.4	
4.5-6	0.5	0.2	0.3	0.5	0.6	0.8	0.9	1.1	1.2	1.4	1.6	
3-6	2.0	0.2	0.3	0.5	0.7	0.8	1.0	1.2	1.4	1.5	1.7	26-30
3-4	0.5	0.2	0.4	0.5	0.7	0.9	1.1	1.3	1.4	1.6	1.8	
4.5-6	0.5	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0	

In the use of 1 cc. samples, as in the writer's serum method, the error resulting from such loss of sample would be significant. To avoid this error, a technique of transfer with washing, such as that

TABLE III

Factors for Calculations, from Observed P_{N_2} Measurements, of Precise and Routine Gasometric Urine Chloride Analyses

Temperature	For precise method; factor F for calculating according to Equation 5*	For routine method; dilution = 20, supernatant sample = 2.0 cc.†							
		Normal urine, K = 0.998				Albuminous urine, K = 0.990			
		a = 0.5		a = 2.0		a = 0.5		a = 2.0	
		Factor by which P_{N_2} is multiplied to find							
		mM Cl per liter	Gm. NaCl per liter	mM Cl per liter	Gm. NaCl per liter	mM Cl per liter	Gm. NaCl per liter	mM Cl per liter	Gm. NaCl per liter
°C.									
15	0.03710	0.1862	0.01089	0.745	0.0436	0.1877	0.01098	0.751	0.0439
16	0.03696	55	85	42	34	70	94	48	38
17	82	48	81	39	32	63	90	45	36
18	68	41	77	37	31	56	86	43	35
19	55	35	73	34	29	50	82	40	33
20	0.03642	0.1828	0.01069	0.731	0.0428	0.1843	0.01078	0.737	0.0431
21	29	22	65	29	26	37	74	35	30
22	16	15	62	26	25	30	71	32	28
23	03	09	58	23	23	24	67	29	26
24	0.03590	02	54	21	22	17	63	27	25
25	0.03578	0.1796	0.01051	0.718	0.0420	0.1811	0.01059	0.724	0.0424
26	66	90	47	16	19	05	55	22	22
27	54	84	44	14	18	0.1799	52	20	21
28	42	78	40	11	16	93	48	17	19
29	30	72	37	09	15	87	45	15	18
30	0.03518	0.1766	0.01033	0.706	0.0413	0.1781	0.01042	0.712	0.0417
31	06	60	30	04	12	75	39	10	15
32	0.03493	53	26	01	10	68	34	07	14
33	81	47	22	0.699	09	62	31	05	12
34	69	41	18	96	07	56	27	02	11

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* For the precise method, under conditions of analysis other than those given above, calculation is made according to Equation 5 directly.

† If supernatant samples of 1.0 cc. are used, results are multiplied by 2. The factors for the routine method have been derived from Equation 5, as described on p. 371 for the conditions indicated above. If urine analyzed by the routine method is calculated to contain less than 60 mM of Cl per liter (or 3.51 gm. of NaCl per liter) correction for $AgIO_3$ solubility must be made, according to Fig. 2 and p. 371.

TABLE IV

Factors for Calculations, from Observed P_{N_2} Measurements, of Gasometric Serum Chloride Analyses, without Deproteinization

Temperature	*Macro*analysis of 0.5 cc. sample		*Micro*analysis of 0.2 cc. sample		*Micro*analysis of 0.1 cc. sample; or *ultramicro*analysis of 0.05 cc. sample		*Ultramicro*analysis of 0.02 cc. sample	
	$d = 20$		$d = 26.05$		$d = 31.05$		$d = 31.15$	
	Supernatant sample = 1.0 cc.						Supernatant sample = 0.5 cc.	
	$a = 0.5$ cc. $K = 0.985$							
	Factor by which P_{N_2} is multiplied to find							
	mM Cl per liter	Mg. NaCl per 100 cc.	mM Cl per liter	Mg. NaCl per 100 cc.	mM Cl per liter	Mg. NaCl per 100 cc.	mM Cl per liter	Mg. NaCl per 100 cc.
°*C.*								
15	0.3780	2.211	0.4925	2.881	0.587	3.434	1.173	6.86
16	66	03	05	70	85	22	69	84
17	52	2.195	0.4890	61	83	11	64	81
18	38	87	70	49	81	3.399	60	79
19	24	79	50	37	79	87	56	76
20	0.3711	2.171	0.4835	2.828	0.577	3.375	1.152	6.74
21	0.3698	63	15	17	75	64	48	72
22	85	56	00	08	73	52	43	69
23	71	48	0.4785	2.799	71	40	39	66
24	58	40	65	88	69	29	35	64
25	0.3646	2.133	0.4750	2.779	0.567	3.317	1.131	6.62
26	34	26	35	70	65	05	27	59
27	22	19	20	61	63	3.294	24	57
28	09	11	00	50	61	82	20	55
29	0.3597	04	0.4685	41	59	70	16	53
30	0.3585	2.097	0.4670	2.732	0.557	3.258	1.112	6.51
31	73	90	55	23	55	47	08	48
32	59	82	35	11	53	35	04	46
33	47	75	20	03	51	23	1.100	44
34	35	68	05	2.694	49	12	1.096	41

The factors have been derived from Equation 5.

TABLE V

Factors for Calculations, from Observed P_{N_2} Measurements, of Gasometric Chloride Analyses of Serum and Whole Blood Filtrates

Temperature	Tungstic acid serum filtrate		Zinc hydroxide serum filtrate		Tungstic acid blood filtrate; *macro*analyses of 1 cc. sample or *micro*analyses of 0.2 cc. sample	
	$d = 10$		$d = 11$*		$d = 25$	
	Supernatant sample = 2.0 cc.†				Supernatant sample = 1.0 cc.	
	$a = 2.0$				$a = 0.5$	
	$K = 1.017$		$K = 0.995$		$K = 1.000$	
	Factor by which P_{N_2} is multiplied to find					
	mM Cl per liter	Mg. NaCl per 100 cc.	mM Cl per liter	Mg. NaCl per 100 cc.	mM Cl per liter	Mg. NaCl per 100 cc.
°C.						
15	0.3655	2.138	0.4110	2.404	0.4656	2.724
16	42	31	0.4094	2.395	38	13
17	28	22	79	86	21	03
18	14	14	63	77	03	2.693
19	01	07	49	69	0.4587	83
20	0.3588	2.099	0.4034	2.360	0.4571	2.674
21	76	92	20	52	54	64
22	63	84	05	43	38	55
23	50	77	0.3991	35	22	45
24	37	69	77	27	05	35
25	0.3525	2.062	0.3963	2.318	0.4490	2.627
26	14	56	50	11	75	18
27	02	49	37	03	60	09
28	0.3490	42	23	2.295	45	00
29	78	35	10	87	30	2.592
30	0.3466	2.028	0.3897	2.280	0.4415	2.583
31	54	21	84	72	00	74
32	42	14	69	63	0.4384	65
33	30	07	56	56	69	56
34	18	00	43	48	54	47

The factors have been derived from Equation 5.

* If the serum filtrate + H_3PO_4 mixture is prepared by diluting 9 volumes of filtrate to 10 volumes (see foot-note 13), $d = 11.11$ and the factors are multiplied by 1.010.

† If supernatant samples of 1 cc. are used, the results are multiplied by 2.004. If supernatant samples of 0.5 cc. are used, the P_{N_2} measurements are made at $a = 0.5$ cc. volume, and the results are multiplied by 0.998.

prescribed for the ultramicroanalysis of serum, would have to be used. The amount of air in the blank would then be increased, as would the time necessary to carry out an analysis. The writer has preferred to use the mercury seal, to determine accurately the loss involved in sampling, and to apply a corresponding correction to the results.

The correction was determined as follows: A sample of 1 N NaOH was taken in the pipette to be used for iodate determinations. The tip was *thoroughly* washed in a stream of distilled water, and the sample was delivered into the chamber through the mercury seal in the cup, as described above. The mercury was run down nearly to the capillary of the cup. The layer of water above was stirred with a rod, then titrated with 0.01 N HCl, with alizarin sulfonate as indicator. The results varied slightly, depending on the combination of pipette and apparatus used. A loss of from 0.003 to 0.005 cc. was found.

In the forms of extraction chambers used for this work the cups were sealed onto the capillary in such a manner that the bottom of the cup was relatively flat and not pointed. The rubber tip on the pipettes used was also flat bottomed, so that it fitted snugly to the cup at the point where the capillary suddenly widened out. Under these conditions the minimum losses mentioned above were observed.

The results of the present paper have been corrected for these errors. In order that a separate calculation may be avoided, an average correction factor m of 1.004 and 1.002, for samples of 1 and 2 cc., respectively, has been incorporated in the tables of factors (Tables III, IV, and V) for use with this method. However, it is strongly recommended that the analyst determine for himself the error involved in the use of any particular pipette and extraction chamber. Should the sample loss be sensibly different from that given above, a further correction should be applied to all results calculated according to Tables III, IV, and V. Thus, if it be found that there is a 0.7 per cent loss on delivery of a 1 cc. sample, all of the results should be increased by a factor of 1.003 over the values calculated according to the appropriate table of factors.

Derivation of Factors of Tables III, IV, and V—The symbols used in the following equations are:

$P =$ mm. N_2 pressure measured in the gasometric iodate analysis of the supernatant fluid from the $AgIO_3$ precipitation of chloride

$a =$ cc. volume at which P is read in the extraction chamber

$s =$ " " of supernatant sample analyzed

$S =$ " total volume of solution extracted in the extraction chamber

$[N_2] =$ mM nitrogen per liter of supernatant sample, found by iodate analysis

$[IO_3]_e =$ mM iodate per liter of original, undiluted sample found by analysis of supernatant sample

$[Cl]_t =$ mM chloride per liter of original, undiluted sample

$f =$ factor converting values of P to values of $[N_2]$, when $s = 1.0$, $S = 3.5$, and $a = 2.0$

$F = f/3$

$d =$ dilution factor, for the number of times the original sample volume was increased by dilution, before being shaken with $AgIO_3$

$m =$ correction factor, for loss of sample in transfer through mercury seal

$K = \frac{[IO_3]_e}{[Cl]_t} =$ empirical reaction yield factor expressing ratio of the concentration of iodate found by analysis to the concentration of chloride originally present, per liter of original sample

From Table III of Van Slyke and Neill (1924) the mM of N_2 obtained from the analysis of the supernatant iodate-containing solution may be calculated. We may use as f their factor for nitrogen from a 1 cc. sample, with solution volume $S = 3.5$ cc., and with the gas measured at the 2.0 cc. volume ($a = 2.0$ cc.).[14] Then, with N_2 measured at any volume, a, mM of N_2 per liter of supernatant sample analyzed will be

$$[N_2] = \frac{P \times f \times a \times m}{2 \times s} \tag{3}$$

To calculate iodate from N_2, the factor $\frac{2}{3}$ is introduced, since 2 moles of iodate yield 3 moles of N_2 (Equation 2). Furthermore, the dilution factor d is used to calculate values in terms of concentrations in the original, undiluted sample.

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[14] A change in value of S, the total amount of liquid in the chamber when the nitrogen gas is evolved, has but slight effect on these calculations (0.1 per cent difference between $S = 2.5$ and 5.0), and hence may be neglected. However, S must always be exactly the same when p_0 is determined in the blank analysis as when p_1 is determined in the iodate analysis.

$$(4)\qquad [IO_3]_e = \frac{2}{3} \times \frac{P \times f \times a \times m \times d}{2 \times s} = \frac{f}{3} \times \frac{Pamd}{s}$$

To calculate the chloride from the iodate values, it is necessary to introduce the factor K, which is slightly less than unity, for the reason that under the usual conditions of analysis 1 mole of chloride yields slightly less than 1 of iodate. For pure chloride solutions under such conditions the value of K has values both theoretically and empirically of from 0.996 to 1.000 (Tables VI and XI). For urine, plasma, and plasma or blood filtrates, however, K has a slightly different, empirical value, which is used in the calculations for each of these materials, and which has been

TABLE VI

Theoretical Calculations of Concentrations at Equilibrium of the Reaction in Water: $AgIO_3 + NaCl \rightleftarrows NaIO_3 + \downarrow AgCl$

$[IO_3^-]_e$ = mM total iodate in solution per liter at end of reaction. $[C^-l]_i$ = mM chloride per liter, *initial*, at beginning of reaction. $[Cl^-]_e$ = mM chloride per liter, *final*, at end of reaction. $[IO_3^-]_s$ = mM iodate per liter, as physically dissolved $AgIO_3$, at end of reaction.

$[IO_3^-]_e$	From Equation			Ratio $\frac{100[IO_3^-]_e}{[Cl^-]_i}$
	8	7	16	
(1)	$[Cl^-]_e$ (2)	$[IO_3^-]_s$ (3)	$[Cl^-]_i$ (4)	(5)
1.00	0.0043	0.0400	0.964	103.70
1.25	0.0054	0.0320	1.223	102.17
1.50	0.0065	0.0267	1.480	101.36
2.00	0.0086	0.0200	1.989	100.57
2.50	0.0108	0.0160	2.495	100.21
3.00	0.0129	0.0133	3.000	100.00
3.50	0.0151	0.0114	3.504	99.90
4.00	0.0173	0.0100	4.007	99.82
5.00	0.0216	0.0080	5.014	99.73
6.00	0.0259	0.0067	6.019	99.68
8.00	0.0345	0.0050	8.030	99.63
10 00	0.0432	0.0040	10.039	99.61
12.00	0.0518	0.0033	12.048	99.60

incorporated in the computation of the factors of Tables III, IV, and V. Therefore, the mM of chloride per liter of *original* sample will be

$$(5) \qquad [\mathrm{Cl}]_i = P \times \left(\frac{Famd}{K_8}\right)^*$$

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* If values in terms of gm. of NaCl per liter are desired, the values of mM of chloride per liter are multiplied by the factor 1/17.1 or 0.0585. In terms of mg. of NaCl per 100 cc., values of mM of chloride per liter are multiplied by 5.85.

The calculated values for F are given in Table III. From these values, with the aid of Equation 5, values for chloride content of samples analyzed under any of the conditions outlined may be calculated. Obviously, from Equation 5 it is possible to combine the various factors for any one set of conditions and thus to calculate a *single* factor (enclosed in parentheses) by which to multiply the nitrogen pressure to obtain chloride concentrations directly. Such single, composite factors are given in Tables III, IV, and V for use with urine, plasma, or protein-free filtrate analyses.

Example—A sample of 0.5 cc. of *serum* was diluted to 10 cc. before addition of silver iodate. Of the centrifuged, supernatant liquid, 1 cc. was delivered through a mercury seal, into the extraction chamber. The p_1 reading measured at 0.5 cc. volume was 371.5 mm. at 25°. The blank p_0 reading was 96.1 mm. at 24.5°. According to Table II the corrected p_0 reading was 96.8 mm. at 25°. Thus $p_1 - p_0 = P = 274.7$ mm. According to Equation 5 the chloride concentration of the original sample is calculated to be

$$\frac{274.7 \times 0.03578 \times 0.5 \times 1.004 \times 20}{0.985 \times 1.0} = 100.2 \text{ mM Cl per liter}$$

The simple calculation by the use of the composite factor for these conditions (Table IV, second column) is merely 274.7 × 0.3646 = 100.2 mM of Cl per liter.

Correction for Solubility of $AgIO_3$ in Routine Method for Urine—As is noted in the following "Theoretical" and "Experimental" sections, the effect on the results of the solubility of $AgIO_3$ becomes significant when the iodate concentration of the supernatant fluid in the chloride analysis is less than 3 mM. In the *routine* analysis of urine, when samples containing less than 60 mM of chloride per liter (or 3.51 gm. of NaCl per liter) are diluted 20-fold, a solubility correction for $AgIO_3$ must, therefore, be applied to the results after calculation according to Table III. These corrections are given in Fig. 2, where the curves indicate the corrections to be subtracted from any given total chloride value obtained by the *routine* procedure. *The curves of Fig. 2 apply only to urine diluted 20-fold with 0.17 M H_3PO_4.* The derivation of the correction curves is discussed in the "Experimental" section following.

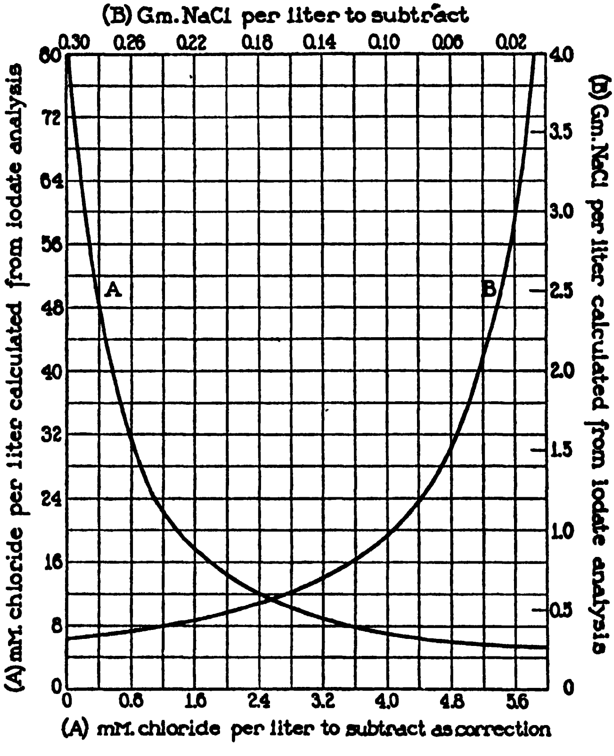

FIG. 2. Corrections to be subtracted from the results of routine urine chloride analyses when calculation according to Table III indicates less than 60 mM of chloride per liter (or 3.2 gm. of NaCl per liter) for the undiluted urine.

Example—A sample of nephritic urine was found by *routine analysis* and calculation from Table III to contain apparently 12.00 mM of chloride per liter. Since the value was less than 60 mM (and therefore less than 3 mM for the diluted analyzed sample), the correction of Curve A of Fig. 2 was applied. The corrected chloride concentration of the undiluted urine was, therefore, 12.00 − 2.40 = 9.60 mM per liter.

THEORETICAL

Upon the completion of the reaction upon which this method is based (Equation 1), a solution will be in equilibrium with both solid silver chloride and solid silver iodate. According to the law of mass action, with complete dissociation of the dissolved silver salts, the relationships for the equilibria will be

$$[Ag^+] \times [Cl^-] = K_{AgCl} \tag{6}$$

$$[Ag^+] \times [IO_3^-] = K_{AgIO_3} \tag{7}$$

where the brackets indicate concentrations in mM per liter, and K_{AgCl} and K_{AgIO_3} indicate the solubility product constants.

In the present method the reaction takes place in acid solutions, usually of 0.085 M phosphoric acid. If the possible effect on solubility of the acidity and other changes in the chemical composition of biological fluids be neglected, one may approximately calculate solubility products in such a medium from the available values for solubility in water. From Brown and MacInnes (1935) the solubility of silver chloride in water at 25° is 0.01314 mM per liter. From the Landolt-Börnstein-Roth "Tabellen" (1912) the solubility of silver iodate at that temperature is 0.19 mM per liter. From our own determinations at the same temperature, this value is 0.20 mM per liter.

From the above values one obtains at 25° values of $K_{AgCl} = (0.01314)^2 = 0.00017266$, and $K_{AgIO_3} = (0.200)^2 = 0.04$. Substituting in Equations 6 and 7, and combining, one obtains

$$\frac{[Cl^-]_e}{[IO_3^-]_e} = \frac{K_{AgCl}}{K_{AgIO_3}} = 0.004316 \tag{8}$$

as the expression for the relative final concentrations of chloride and iodate ion in water in equilibrium with both solid silver salts.

In the reaction of Equation 1 the difference between the initial chloride concentration, $[Cl^-]_i$, and the final, $[Cl^-]_e$, is equal to the concentration of iodate, $[IO_3^-]_r$, dissolved from silver iodate by reaction with chloride. Thus

$$[Cl^-]_i = [Cl^-]_e + [IO_3^-]_r \tag{9}$$

Also, the iodate dissolved as the result of reaction of $AgIO_3$ with Cl is joined in the solution by a slight amount of iodate which dissolves physically as $AgIO_3$, and is indicated as $[IO_3^-]_s$. Thus

$$[IO_3^-]_e = [IO_3^-]_r + [IO_3^-]_s \tag{10}$$

From Equations 9 and 8

$$\frac{[Cl^-]_i}{[IO_3^-]_e} = \frac{[IO_3^-]_r + [Cl^-]_e}{[IO_3^-]_e} = \frac{[IO_3^-]_r}{[IO_3^-]_e} + 0.004316 \tag{11}$$

From Equation 10 one obtains, for the relationship between the initial chloride and the final total iodate concentrations,

$$\frac{[Cl^-]_i}{[IO_3^-]_e} = \frac{[IO_3^-]_r}{[IO_3^-]_r + [IO_3^-]_s} + 0.004316 \tag{12}$$

As the concentration of iodate, $[IO_3^-]_r$, from chloride increases, the amount of iodate from physically dissolved silver iodate, $[IO_3^-]_s$, decreases, until a point is reached where $[IO_3^-]_s$ becomes vanishingly small. Equation 12 then becomes

$$[Cl^-]_i = 1.004316[IO_3^-]_e \tag{13}$$

At this point then,

$$[IO_3^-]_e = [IO_3^-]_r = [Cl^-]_i - [Cl^-]_e = 0.9957[Cl^-]_i \tag{14}$$

which means that the *limiting* value for the extent of the reaction of Equation 1, or the yield of iodate from reaction with chloride, will be reached when 99.57 per cent of the chloride initially in solution has been precipitated, with a corresponding liberation of iodate, mole for mole.

However, this condition is *apparently* exceeded, since in all cases there is *some* solubility of silver iodate, depending on the amount of iodate released by the chloride. Since, under these conditions, from Equations 9 and 10,

$$[\mathrm{Cl}^-]_i = [\mathrm{Cl}^-]_s + [\mathrm{IO}_3^-]_e - [\mathrm{IO}_3^-]_s \tag{15}$$

and since $[\mathrm{IO}_3^-]_s$ can only be derived from silver iodate with solution of the *same* number of silver $[\mathrm{Ag}^+]_s$ ions per liter, and since $[\mathrm{Ag}^+]_s$ is defined by Equation 7, we may calculate values for $[\mathrm{Cl}^-]_i$ entirely in terms of $[\mathrm{IO}_3^-]_e$ to obtain another expression for Equation 11 or 12 which can be used for calculating the relationships between the initial chloride and the final total iodate concentration. That expression is

$$[\mathrm{Cl}^-]_i = 1.004316[\mathrm{IO}_3^-]_e - \frac{0.04}{[\mathrm{IO}_3^-]_e} \tag{16}$$

From Equation 15 values for $[\mathrm{Cl}^-]_i$, corresponding to analyzed values of $[\mathrm{IO}_3^-]_e$, have been calculated for Table VI. The effect of the added iodate in the form of $[\mathrm{IO}_3^-]_s$ is shown in Table VI in the values calculated for the ratio we refer to as the reaction yield factor K, or $[\mathrm{IO}_3^-]_e/[\mathrm{Cl}^-]_i$.

The *apparent* yield of iodate, $[\mathrm{IO}_3^-]_e$, from chloride solutions of between 12 mM and 3 mM per liter would be from 99.61 to 100.00 per cent, a variation accounted for by the increase in physically dissolved silver iodate of from 0.04 to 0.44 per cent of the total iodate in the solution at equilibrium. At concentrations below 3 mM per liter of chloride the correction for dissolved $[\mathrm{IO}_3^-]_s$ becomes significant. At 1 mM per liter of chloride in aqueous solutions it amounts to 4 per cent of the total $[\mathrm{IO}_3^-]_e$ present at the end of the reaction.[15]

The foregoing theoretical calculations are based on the solubilities of silver iodate and chloride *in water* at equilibrium. In another solvent, such as 0.085 M or 0.17 M phosphoric acid dilutions of salt or other solutions, the solubilities of AgCl and $\mathrm{AgIO_3}$, and consequently the solubility product ratios, may be expected to be somewhat different. Furthermore, temperature variation may also be a factor. The available data indicate that the relative solubilities of AgCl and $\mathrm{AgIO_3}$ are little changed with variation in temperature. We have no reliable, accurate data on the effect of 0.085 M and 0.17 M $\mathrm{H_3PO_4}$ on these *relative solubilities*. Our own data indicate that the solubility of $AgIO_3$, itself, is approximately 30 per cent greater in 0.17 M H_3PO_4 than in water.

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[15] For the exceptional cases in which solutions with chloride of less than 3 mM concentration may be encountered, see the "Experimental" section and the section on "Calculations," with especial reference to the *routine method* for urine.

However, it does not appear that the differences between the calculated and the real values for relative solubilities, under the usual analytical conditions of the present method, are significant. A change of 25 per cent in the value of the ratios of Equation 8 would result in a calculated yield of iodate from chloride, for chloride solutions between 12 and 3 mM, only 0.1 to 0.3 per cent different from that given by Equation 16. The experimental results bearing on the extent of the reactions involved, and on the question of correcting for dissolved iodate, will be considered in the following sections.

EXPERIMENTAL

Solubility of Silver Iodate—The determination of the solubility in water is suggested ("Reagents") as one of the two tests of the purity of the silver iodate. The experiments done in this connection are detailed in the following. Saturated silver iodate solutions were prepared by shaking 15 cc. portions of water with $AgIO_3$ for 30 minutes at 25°. The solutions were filtered or centrifuged. The dissolved iodate was determined both gasometrically and by titration.

For the *gasometric* determination, 5 cc. of hydrazine and 5 cc. of filtrate were measured into the extraction chamber. The chamber was evacuated and the solution was shaken for 3 minutes. The gas pressure was then read at the 0.5 cc. volume for p_1. The p_0 reading was obtained in a blank analysis in which water replaced the saturated solution of $AgIO_3$. A pressure of about 51 mm. of N_2 from dissolved $AgIO_3$ was obtained.

Titrimetrically (Sendroy, 1937, *a*) a more accurate result was obtained by titrating 3 cc. samples of filtrate with 0.0012 N $Na_2S_2O_3$, of which about 3 cc. were required. Before the titration was begun, 5 drops of 0.85 M H_3PO_4 and 1 drop of 5 per cent KI were added. Near the end-point 1 drop of 0.5 per cent starch solution was added. The thiosulfate solution was standardized with 0.0012 N KIO_3 (= 0.0002 M) in place of the sample.

The results of Table VII indicate that under these conditions, the solubility of silver iodate in water is 0.20 mM per liter, a value that is constant for saturation obtained by shaking between 30 and 90 minutes. Upon the addition of phosphoric acid, the $AgIO_3$ solubility increased to 0.25 to 0.26 mM per liter in 0.17 M H_3PO_4.[16]

As has been noted in the preceding "Theoretical" section, the solubility of $AgIO_3$ in the supernatant fluid of the chloride analysis is in all cases much less than these values, because the $NaIO_3$ formed depresses the $AgIO_3$ solubility. Within the concentration

TABLE VII

Results of Solubility Determinations in Water and in H_3PO_4 Solutions at 24–25° of Several Lots of $AgIO_3$

Lot No.	Solvent	Time of shaking	Method of iodate determination	Solubility of $AgIO_3$
		min.		*mM per l.*
1	H_2O	30	Gasometric	0.20
		30	"	0.20
		60	"	0.20
		60	"	0.19
		60	"	0.19
2	"	30	"	0.19
		60	"	0.21
		60	"	0.20
		60	"	0.21
		90	"	0.20
3	"	3	Titrimetric	0.19
		30	Gasometric	0.19
		40	"	0.20
3	0.085 M H_3PO_4	3	Titrimetric	0.24
		3	Gasometric	0.23
		30	"	0.23
		40	"	0.23
		40	Titrimetric	0.24
		60	Gasometric	0.24
3	0.128 " "	3	"	0.24
3	0.17 " "	3	"	0.25
		3	"	0.26

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[16] These results were later confirmed by the colorimetric technique (Sendroy, 1937, *b*), the accuracy of which is equal to that of the titrimetric method for the determination of iodate in such dilute concentrations.

limits of 12 and 3 mM chloride, the $AgIO_3$ solubility is negligible, as is indicated by the calculated values for dissolved $AgIO_3$ in aqueous solutions of NaCl (Table VI, Column 3). We have in fact been unable to measure any definite increase in the iodate concentrations of known solutions of KIO_3 in 0.085 M H_3PO_4 of the concentrations formed in serum chloride analysis, when they were shaken with $AgIO_3$ for 3 minutes (Table VIII). Under these conditions, therefore, no correction for $AgIO_3$ solubility is required.

TABLE VIII

Effect of Shaking Iodate Solutions in 0.085 M Phosphoric Acid, with Solid $AgIO_3$ for 3 Minutes at 24–25°. Gasometric Measurements

$AgIO_3$	Iodate		$AgIO_3$ solubility	
	Present in $KH(IO_3)_2$ solution	Found by analysis	Found by difference of averages	Theoretical for unacidified aqueous solution according to Table VI
	mM per l.	*mM per l.*	*mM per l.*	*mM per l.*
None	10.00	10.00		
		10.00		
		10.04		
Added		10.00	−0.01	+0.004
		10.02	+0.01	+0.004
None	2.50	2.500		
		2.503		
		2.497	−0.004	+0.016
Added		2.509	+0.008	+0.016
		2.504	+0.003	+0.016

On the other hand, in more dilute iodate solutions, such as may be obtained in the *routine method* applied to low chloride urines, the solubility of $AgIO_3$ becomes theoretically greater. The effect of this increased solubility on the yield of total dissolved iodate from small concentrations of chloride can be demonstrated experimentally. The results are discussed in connection with the analysis of low chloride nephritic urines.

Determination of Chloride in Known Salt Solutions—In the study of the various factors affecting the reaction of chlorides with silver iodate, several hundred analyses were carried out on solutions of known chloride content. The starting material was always a 0.1 M chloride solution which was diluted from 10 to 30 times for the determinations. Chlorides of reagent grade, obtained from various sources, were used. Some were used directly from the container as purchased, others were dried or recrystallized several times, and one was fused after three recrystallizations. The results with all were found to be the same within the limits of error. Since the large number of analyses made makes it impractical to list all of the results, the tables illustrating the various significant points should be considered as merely representative of a still larger number of results not reported.

Effect of pH—Chlorides are usually precipitated in an acid medium in order to prevent the precipitation of other insoluble silver salts, and to obtain a good coagulation of the precipitated material. In unacidified salt solutions diluted to 10 mM of chloride per liter, the silver chloride, at least, remained partially in colloidal form and caused slightly high results. On the other hand, nitric acid, in the presence of which chlorides are usually precipitated, could not be used for work in the Van Slyke apparatus, since it reacts with mercury.

Table IX indicates the results of a large number of determinations made in a variety of diluents, as a test of their suitability for work with biological materials. In duplicate analyses two separate portions of standard chloride solution were shaken with $AgIO_3$. The supernatant liquid in all cases, except when water alone was the diluent, was almost clear. In inorganic chloride solutions of pH less than 6.5 phosphates were not found to interfere with the reaction between chloride and silver iodate. Of other substances added, only bicarbonate in solutions above pH 6.2 was found to interfere; it caused solution of 1.008 moles of iodate per mole of chloride. All of the other results were close to the theoretically calculated yield of 99.6 per cent (Equation 15) for 0.01 M unacidified aqueous chloride solutions.

Amount of Silver Iodate Required—By calculation, 10 cc. of 0.01 M chloride solution require 0.0283 gm. of $AgIO_3$. In order to provide a sufficient excess, experiments were carried out to determine the iodate yield from 0.01 M solutions of NaCl in 0.085 M H_3PO_4, to which different amounts of $AgIO_3$ were added. The results are as follows:

$AgIO_3$ per 10 cc. solution, *gm*	0.03	0.06	0.12
IO_3 per liter found, *mM*	9.48	10.00	9.98

From this it is apparent that twice the calculated necessary amount of $AgIO_3$ was adequate for complete reaction (theoretical yield in acidified solution = 99.6 per cent). Our procedure calls for the use of what is equivalent to at least 3 times the stoichiometrical amount. Use of more has no further effect other than to make the supernatant liquid slightly clearer in some solutions.

TABLE IX

Effect of pH on Silver Iodate Determination of Chloride in 0.01 M Solutions of Sodium Chloride at Room Temperature. Gasometric Results

No. of solutions analysed	Diluent in which NaCl was present as 0.01 M	pH	Average chloride concentration found by analysis
			mM per l.
4	Water	6.6	10.01
5	0.1% acetic acid	3.7	9.99
3	0.5% " "	3.2	9.96
2	M/15 acetate mixture	4.0	9.95
2	M/15 " "	4.5	9.97
2	M/15 " "	5.0	10.01
2	M/15 " "	5.5	10.00
89	0.085 M H_3PO_4	2.1	9.98
3	0.17 " "	2.0	9.98
20	Tungstic acid mixture	2.4	9.99
46	M/15 KH_2PO_4	4.5	9.98
4	M/15 phosphate mixture	5.5	9.98
4	M/15 " "	6.5	10.01
4	M/15 " "	7.5	10.00
4	M/15 " "	8.5	10.01
6	M/15 phosphate + M/15 $NaHCO_3$	6.2	10.07
4	M/15 " + M/15 "	6.8	10.09

Time Necessary for Completion of Reaction of $AgIO_3$ with Chloride—Solutions of 0.1 M NaCl diluted to 10 to 40 times with 0.085 M H_3PO_4 were shaken with $AgIO_3$ (0.1 gm. per 10 cc. of solution) for varying lengths of time, from ½ minute to 1 hour. Table X shows that the reaction was nearly complete in ½ minute.

The recommended procedure, of a minimum 2 minutes shaking with $AgIO_3$, allows a wide margin of safety for the completion of the reaction when the chloride concentration is between 2.5 and 10 mM.

Effect of Temperature on Reaction of $AgIO_3$ with Chloride—Solutions of 0.1 M NaCl were diluted to 10 times with 0.085 M phosphoric acid, then brought to thermal equilibrium by shaking in tubes kept at different constant temperatures. Then $AgIO_3$ was introduced, and the shaking was continued at the same temperatures, for times ranging from ½ to 10 minutes. Repeated experiments carried out at temperatures ranging from 15–35° failed to show any consistent effect of temperature on the reactions.

TABLE X

Effect of Time of Shaking with $AgIO_3$ on the Reaction: $NaCl + AgIO_3 \rightleftarrows \downarrow AgCl + NaIO_3$ As Found by Gasometric Measurements

Experiment No.	Chloride concentration	Time of shaking with $AgIO_3$							Theoretical ratio $\frac{100[IO_3]_e}{[Cl]_i}$ for unacidified aqueous solutions
		½ min.	1½ min.	3 min.	5 min.	10 min.	30 min.	60 min.	
		Extent of reaction as indicated by ratio, $100 \times \frac{\text{mM per liter iodate found}}{\text{mM per liter chloride present}}$							
	mM per l.								
1	10.00				99.9	100.0			99.6
2	10.00				99.3	99.9	99.8	99.6	
3	10.00		99.7	99.3					
4	10.00				99.9	99.9	99.7	99.9	
5	10.00	99.5	99.5						
6	10.00	99.4	99.6						
7	5.00		99.5		99.4	99.8	100.2	99.6	99.7
8	5.00	99.5	100.0						
9	5.00	99.5	99.6						
10	2.50		100.5		100.3	100.2	100.3	99.8	100.2
11	2.50			99.9	99.7				
12	2.50		99.7	100.4			99.5	99.8	

The results of a typical experiment with a 10 mM chloride solution were as follows:

Temperature, °*C*......	15	20	25	33	35
Iodate per liter found, *mM*..........	9.98	9.99	9.99	9.94	9.96

No solubility data are available for the calculation of the equilibrium (Equation 8) at various temperatures other than at 20° and 25°. Theoretically, the difference between the iodate yield, relative to the initial chloride concentration, at 20° and at 25° is about 0.1 per cent. Within ordinary limits of room temperature, therefore, the effect of variations in the temperature may be considered negligible.

TABLE XI

Summary of Results of Chloride Determinations in 0.085 M Phosphoric Acid Solutions of Known Composition, at Varying Dilutions

Chloride present = $[Cl]_i$ (1)	Method of iodate analysis (2)	No. of solutions analysed (3)	Measured yield of total iodate from chloride, ratio $\frac{100[IO_3^-]_e}{[Cl^-]_i}$			
			Range (4)	Mean value of all results (5)	Average deviation from mean (6)	Theoretical value for unacidified aqueous solution (Table VI) (7)
mM per l.						
10.00	Gasometric	89	99.1–100.5	99.8	±0.26	99.6
	Titrimetric	5	99.2– 99.9	99.5	±0.24	
	(Colorimetric)		(99.2– 99.6)	(99.4)		
8.00	Gasometric	11	99.2– 99.9	99.6	±0.23	99.6
7.50	"	4	99.6–100.0	99.8	±0.18	99.6
6.00	"	6	99.2–100.4	99.9	±0.30	99.7
5.00	"	24	99.3–100.4	99.8	±0.24	99.7
	Titrimetric	8	99.3–100.0	99.6	±0.20	
	(Colorimetric)		(99.2–100.0)	(99.6)		
4.00	Gasometric	37	99.3–100.5	100.0	±0.24	99.8
	Titrimetric	29	99.1–100.4	99.8	±0.24	
	(Colorimetric)			(100.5)		
3.00	Gasometric	8	99.3–100.3	100.0	±0.24	100.0
	Titrimetric	15	99.4–100.4	100.0	±0.23	
2.50	Gasometric	8	100.0–101.1	100.2	±0.44	100.2
	(Colorimetric)		(99.6–100.0)	(99.8)		
2.00	Gasometric	10	100.0–101.0	100.5	±0.26	100.6

Effect of Variation in Chloride Concentration on the Reaction: $AgIO_3 + NaCl \rightleftarrows \downarrow AgCl + NaIO_3$—From theoretical considerations, with decrease in chloride concentration one may expect an increased physical solubility of silver iodate. With sufficient dilution of the chloride, the physically dissolved $AgIO_3$ added to the formed $NaIO_3$ is sufficient to increase measurably the amount of total iodate dissolved per mole of chloride (Table VI). The actual $AgIO_3$ solubility itself, since it is so small (Table VI, Column 3) under these conditions, is a difficult thing to demonstrate (Table VIII) in any simple analysis of chloride solutions between 2.5 and 10 mM, but its *increase* with decrease in chloride concentration becomes apparent in the average results of a large number of analyses, as is shown by the results of Table XI. In the previous "Theoretical" section it was also pointed out that the increase in solubility of $AgIO_3$ in acid medium over that in neutral water would result in a slight increase of values for the moles of total iodate obtained per mole of chloride. In fact, the results of Table XI (Column 5) average slightly more than 0.1 per cent higher than the corresponding calculated values for the same concentrations of chloride in unacidified water solutions (Column 7).

TABLE XII

Distribution of Some Results of Table XI for Gasometric and Titrimetric Analyses of Known Chloride Solutions in 0.085 M H_3PO_4

Chloride concentration	No. of solutions analyzed	Mean concentration of dissolved iodate found	Percentage distribution of results for total dissolved iodate found for limits of variation from mean, of					
			±0.0%	±0.1%	±0.2%	±0.3%	±0.5%	±0.7%
mM per l.		*mM per l.*						
10.00	94	9.98	11	36	54	71	95	100
4.00	66	3.996	15	47	64	74	93	100

The results of Table XI, which include only determinations of chloride in phosphoric acid solutions, are indicative of the reliability of the method for inorganic salt solutions. The average deviation from the mean for the measured values of Column 5 is about ±0.24 per cent for both the *gasometric* and *titrimetric* (Sendroy, 1937, *a*) determinations. The *colorimetric* (Sendroy, 1937, *b*) results have shown a mean variation of about ±1.0 per cent of the theoretical, with limits of ±2.0 per cent.

The distribution of results of the two larger groups of analyses is shown in Table XII.

Effect of Various Substances on Reaction of $AgIO_3$ with Chloride

TABLE XIII

Effect of Various Substances on Reaction of $AgIO_3$ with Chloride or Other Halides

Group No.	No. of solutions analysed	Composition of diluted halide solution	pH of diluted halide solution	Method of iodate analysis*	Total dissolved iodate from halide, ratio $\frac{100[IO_3]_e}{[\text{halide}]_i}$	
					Found by analysis	Theoretical in unacidified halide solution
1	2	10 mM KBr, 0.085 M H_3PO_4	2.1	G.	100.2	100.0
	2	4.895 mM KBr, 0.168 mM KCl, 0.085 M H_3PO_4	2.1	"	99.9	100.0
2	4	10 mM KI, 0.061 M KH_2PO_4	4.5	"	100.0	100.0
	2	1 " " 9 mM NaCl, 0.085 M H_3PO_4	2.1	"	95.7	99.6
	2	1 " " 9 " " 0.085 " "	2.1	T.	95.7	99.6
	2	0.50 mM KI, 9.50 mM NaCl, 0.085 M H_3PO_4	2.1	G.	98.5	99.6
	2	0.25 " " 9.75 " " 0.085 " "	2.1	"	99.2	99.6
3	4	10 mM NH_4Cl, 0.061 M KH_2PO_4	4.5	"	99.0	99.6
4	2	10 " HCl, 0.2 M acetate mixture	3.9	"	99.6	99.6
	6	10 " " 0.01 " KH_2PO_4, 0.085 M H_3PO_4		"	99.6	99.6
	2	10 " " 0.085 " H_3PO_4		T.	99.5	99.6
	2	5 " " 0.085 " "		"	99.6	99.7
5	2	10 " NaCl, 2 mM $CaCO_3$, 0.17 M H_3PO_4	2.0	G.	99.9	99.6
6	2	10 " " 2 " $Na_2C_2O_4$, 0.17 M H_3PO_4		"	99.6	99.6
	2	5 " " 0.05% $K_2C_2O_4$, 0.085 M H_3PO_4		"	99.9	99.7
	2	5 " " 0.05% " 0.085 " "		T.	100.0	99.7
7	2	5 " " 0.05% Na citrate, 0.085 M H_3PO_4		G.	100.0	99.7
	2	5 mM NaCl, 0.05% Na citrate, 0.085 M H_3PO_4		T.	100.0	99.7
8	2	5 mM NaCl, saturated uric acid, 0.085 M H_3PO_4		G.	100.2	99.7
	2	4 mM NaCl, saturated uric acid, 0.085 M H_3PO_4		T.	99.8	99.8
	2	4 mM NaCl, saturated uric acid, 0.085 M H_3PO_4		G.	100.0	99.8
9	4	5 mM arginine hydrochloride, 0.085 M H_3PO_4		"	99.6	99.7
	4	5 mM NaCl, 5 mM alanine, 0.085 M H_3PO_4		"	100.2	99.7
	4	5 " " 5 " glycine, 0.085 " "		"	100.2	99.7
10	4	10 " " 0.1% glucose, 0.085 M H_3PO_4		"	99.8	99.6

* G. = gasometric; T. = titrimetric.

or Other Halides—The data of Table XIII supplement those of Table IX. The following conclusions are apparent. Bromides may be analyzed quantitatively by the same technique as for chloride (Group 1). Since silver bromide is much more insoluble than chloride, the reaction of bromide with $AgIO_3$ may be expected, according to solubility product relationships, to go even more nearly to completion, so that the yield of iodate per mole of bromide is 1.000 mole instead of the 0.996 mole per mole of chloride. As calculated from AgI solubility in water, the same is true for iodide. However, the experimental results show that iodides (Group 2) in this reaction are extremely sensitive to strong acid, with the result that the iodate found in solution is low, if the pH is much below 4.5. As has been shown, chlorides in inorganic salt solutions (and presumably bromides, also) in their reaction with $AgIO_3$ are unaffected by pH changes over a wide range (Table IX). When iodide is present in a mixture of halides, it is desirable to keep the pH at 4.5. On the other hand, this is too high a pH for the analysis of chloride in biological material by this method. In such solutions the presence of iodide at pH 4.5 may give rise to slightly high total halide results. Iodide is rarely present, however, in urine or plasma in amounts sufficient to necessitate any departure from the prescribed technique of diluting with phosphoric acid.

Other results of Table XIII indicate that the ammonium ion (Group 3) is without effect, and that hydrochloric acid may be standardized simply by diluting with 0.085 M H_3PO_4 and analyzing titrimetrically or gasometrically (Group 4).

The presence of calcium, oxalate, citrate, uric acid, glucose, and some amino acids, in concentrations exceeding several times the amounts usually present in urine or plasma when diluted with phosphoric acid according to the prescribed procedure, showed no significant effect on the reaction of chloride with $AgIO_3$. The variation in results was within the limits found for the results of Table IX.

Procedures Used in Testing Applicability of the Silver Iodate Method to Blood and Urine

The method has been tested in two ways: (1) Its results have been compared with those of chloride methods that have been accepted as standard procedures in general use. (2) In the case of plasma, the material has been freed of halogens by dialysis, and known amounts of NaCl have then been added. Chlorides were determined by the silver iodate method. Comparison of the results with the known amounts of NaCl added, afforded, for the accuracy of the iodate method, a test which was independent of the exactness of any other analysis. This rigid test was applied by Van Slyke (1923-24) to the nitric acid digestion method for plasma chloride, but so far as the writer is aware, with the exception of the colorimetric procedure of Westfall, Findley, and Richards (1934), it has not been applied to any of the other numerous blood chloride methods that have appeared in the literature.

Comparison with Chloride Determined by Standard Methods—Two standard chloride methods have been used, those of Van Slyke (1923-24) and of Sunderman and Williams (1933). In the Van Slyke method a standard solution of $AgNO_3$ in concentrated HNO_3 is added to the chloride-containing fluid, the organic matter is destroyed by digestion at 100° or by boiling, and the excess silver is titrated with sulfocyanate by Volhard's procedure, under the conditions adopted by Whitehorn (1920-21). In Sunderman and Williams' modification the same nitric acid digestion in the presence of $AgNO_3$ is used; but it is preceded by digestion in alkali to prevent the possibility that any chloride may be occluded by fat particles resistant to HNO_3. Furthermore, the $AgNO_3$ is added in aqueous solution before the HNO_3, as recommended by Wilson and Ball (1928). The Sunderman and Williams method, therefore, involves the successive addition of three reagents in place of the single $AgNO_3$-HNO_3 solution used by Van Slyke.

The author, in agreement with Eisenman (1929), has never encountered any significant error in analysis of plasma or serum by simple Van Slyke technique (see discussion in Peters and Van Slyke (1932) p. 831). The results were usually the same as by the Sunderman-Williams procedure, within the limits of titration error. In some sera the Sunderman-Williams method has given slightly higher results, but never by more than 1 part in 200. For comparison with the silver iodate method of serum analysis, both of the digestion methods have been used.

For some samples of *whole blood* the precautionary additions made by Sunderman and Williams to the Van Slyke procedure raise by as much as 1 per cent the chloride values found; the range of the effect has been from 0.0 to 1.0 per cent. There is no exact proof that the higher values are the more accurate, but it appears probable that they are. For comparison with the iodate method in whole blood we have, therefore, used the Sunderman-Williams technique exclusively.

In *urine*, where there are no significant amounts of lipid material or solid tissues to demand resolution by alkaline digestion, we have used the Van Slyke (1923–24) method for comparison. Harvey's (1910) application of the simple Volhard titration, without preliminary nitric acid digestion, has been found grossly inaccurate in albuminous urines (Sendroy, 1937, *c*).

Recovery of Known Amounts of Chloride Added to Serum Previously Freed of Chloride—This procedure was followed essentially as described by Van Slyke (1923–24) for whole blood, with modifications to adapt it to the iodate method as detailed later in connection with the experiments.

Urine

Normal urines containing 92 to 268 mM of chloride per liter (= 5.4 to 15.7 gm. of NaCl per liter) were diluted, as in the *precise procedure*, 10 to 25 times with 0.17 M H_3PO_4, and shaken with $AgIO_3$. The average yield of total dissolved iodate was 0.998 mole per mole of chloride present, indicated by the values for the ratio $100[IO_3]:[Cl]$ (= K) in Table XIV. In this ratio $[IO_3]$ represents the moles of iodate per liter of urine found in the sample after the reaction with $AgIO_3$, while [Cl] represents moles of chloride per liter of urine, as found by the Van Slyke (1923–24) digestion method.[17] The iodate yields for these 60 urine samples were, therefore, in complete agreement with the value of 0.998 mole of iodate per mole of chloride obtained for the known chloride solutions of Table XI.

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[17] This is consistent with the use of the ratio $[IO]_e : [Cl]_t$, or K, used in the calculations and in the experimental work with known concentrations of chloride. There [Cl], instead of being a value found by a standard method, is the true concentration fixed by the use of known amounts of pure halide salts. Thus, in general, the ratio $100[IO_3]: [Cl]$ represents that percentage of a known or analytically determined amount of halide which is measured as total dissolved iodate by the present method. The average values of K found by the precise method, 0.998 for normal urines, and 0.990 for nephritic urines, have been incorporated in the calculation of the factors of Table III.

When normal urines were analyzed by the *routine procedure*, the average of the results was the same as above. However, the probable accuracy of any individual determination was decreased by the error involved in diluting to the 10 cc. volume mark of the graduated 15 cc. centrifuge tube. These determinations were therefore in error by ±1.7 per cent.

Table XIV

Summary of Results of Comparative Analyses of Chloride in Human Urine by Gasometric and Titrimetric (Sendroy, 1937, a) Procedures, and by the Van Slyke (1923-24) Digestion Method

Samples	No. of samples analysed	Ratio $\frac{100\,[IO_3]^*}{[Cl]}$			Procedure
		Average	Mean deviation from average	Extreme range	
Normal urines	37	99.8	±0.32	98.8–100.5	Gasometric, precise
	13	99.8	±0.38	98.6–100.2	Titrimetric, "
	10	99.8	±1.2	98.6–101.0	Gasometric, routine
Nephritic urines (containing protein)	36	98.9	±0.52	97.9–100.0	" precise
	25	99.1	±0.49	98.0–100.0	Titrimetric, "
	14	99.1	±3.7	92.0–107.7	Gasometric, routine
	7	99.4	±1.9	96.1–102.3	Titrimetric, "

* Ratio, $\frac{[IO_3]}{[Cl]} = \frac{\text{mM iodate per liter urine, found by silver iodate method}}{\text{mM chloride per liter urine, found by Van Slyke digestion method}} = K.$

Nephritic urines (Table XIV), because of salt-restricted diets, contained 8 to 62 mM of chloride per liter (= 0.47 to 3.6 gm. of NaCl per liter). Of these urines, a few which did not contain proteins gave results by the precise procedure like those of normal urines, when compared with the Van Slyke digestion method. The others, of which 61 samples were studied, contained from 2 to 50 gm. of protein per liter. These urines yielded, on the average, 0.989 (gasometric) and 0.991 (titrimetric) mole of iodate per mole of chloride found by the digestion method. The mean deviation from these averages was about the same as for normal urines.

When these low chloride urines were analyzed by the *routine procedure*, the average error of the gasometric or titrimetric determination was about ±3 per cent, with a sample now and then showing a discrepancy of as much as ±7 per cent from the digestion method values. However, *for such low chloride material*, even this extreme error is not significant for most clinical purposes. Furthermore, although for these samples small amounts of silver nitrate and dilute NH_4CNS were used to increase the accuracy of the digestion method, the discrepancies found cannot be attributed entirely to the iodate procedure.

Sources of Error in the Silver Iodate Determination of Chloride in Urine

1. Reducing Effect of Urine on Dissolved Iodate—In the analyses of the urines summarized in Table XIV it was noticed that some very few samples, in both the normal and pathological classifications, gave slight decreasing values on each repeated analysis of the supernatant fluid. Upon further study of thirteen normal and twelve pathological urine specimens analyzed by the routine procedure, it was found that, as time elapsed after the shaking with $AgIO_3$, the iodate content of the supernatant fluid, as it stood over the centrifuged $AgIO_3$ and AgCl, decreased progressively, as shown in Fig. 3. In this respect, there seemed to be no difference between normal or pathological urines, nor could the variation in loss of iodate for different urine samples be ascribed to any known factor. *These experiments show that, if the supernatant fluid in urine analysis is analyzed within 1 hour after centrifugation, no serious error will occur from loss of iodate.*

Evidently, human urine contains material which may gradually destroy iodate under the conditions of analysis. This was further shown when KIO_3 or $KH(IO_3)_2$ was added to the dilute, acidified urine to make the iodate concentration 10 mM. Upon immediate analysis of either pathological or normal urines, 88 to 98 per cent of the added iodate was found. Two pathological urines to which iodate had been added, when analyzed gasometrically after 18 hours, yielded no nitrogen at all upon addition of the hydrazine. The disappearance of iodate in any given sample was more rapid when soluble iodate salt alone was added than when the iodate was obtained in solution by reaction of chloride with solid $AgIO_3$.

When urine is shaken with $AgIO_3$, the dissolved iodate that may be destroyed during the shaking appears to be almost completely replaced by solution of more iodate.[18] By the time the shaking period of 2 minutes is over, the iodate destruction, except in very few cases, has become sufficiently slow, so that it does not diminish the dissolved total iodate concentration significantly during the next hour (Table XIV). Hence, if the iodate determination is carried out within this time after the shaking with $AgIO_3$ is finished, significant error is avoided.

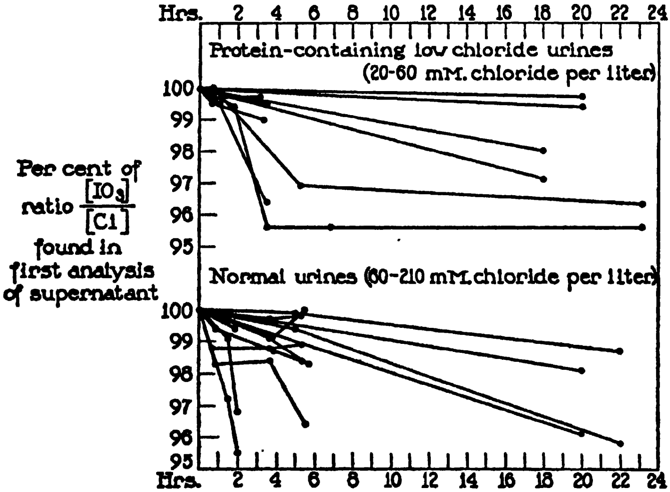

FIG. 3. Disappearance, with time, of dissolved iodate in the chloride determination of normal, and protein-containing, low chloride, nephritic urines.

2. Effect of Physical Solubility of $AgIO_3$ in the Routine Analysis of Low Chloride Urines—In addition to the possible error caused by loss of iodate in the supernatant liquid, another discrepancy which may occur in the analysis of low chloride solutions of any material makes the *routine analysis* of low chloride urines less exact than the *precise analysis.* When the chloride content of a solution is low, the physically dissolved $AgIO_3$ contributes a considerable part to the total iodate found. Furthermore, under these conditions, with 2 minutes allowed for the reaction, equilibrium of the supernatant urine with $AgIO_3$ and AgCl is only approximate. In addition, there may be a considerable variation in the solubility of $AgIO_3$ in different urines, even when diluted 20 times.

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[18] The probable reactions involved are discussed in a later section, p. 398.

It seemed best to determine empirically the apparent solubility correction for $AgIO_3$ in urine samples of varying, low chloride concentration. This was done by comparison of the results by the iodate method of a series of twenty-one different samples of low chloride urine with results by the Van Slyke digestion method. In each case the excess in the value of the iodate result over that by the digestion method was arbitrarily attributed to the solubility effect of $AgIO_3$ in the 20-fold diluted urine (Column 4, Table XV). Fairly wide discrepancies in these values, at the same chloride concentrations, were encountered. However, as might be expected, the average solubility curve (Fig. 2, "Calculations") found best suited for the entire group of the results was very nearly like that calculated for the solubility of $AgIO_3$ in solutions of chloride of these concentrations, dissolved in 0.17 M H_3PO_4. When the corrections of Fig. 2 were applied to this group of urines, results averaged within ±3 per cent of agreement with the digestion values.

That the marked variation of the solubility of $AgIO_3$ in some individual samples may account for larger differences in results is shown by an experiment carried out with Sample 19 of Table XV. This sample contained 41 gm. of protein per liter and gave distinctly positive tests for bile. (1) According to Fig. 2, the physical solubility of $AgIO_3$ in this urine diluted 20-fold, and then containing 0.75 mM of chloride per liter, would be 0.083 mM per liter. (2) From Column 4, Table XV, the apparent solubility found was 0.113 mM per liter of diluted urine or supernatant liquid. (3) When $AgNO_3$ was added to the dilute, acidified urine in concentration of 0.75 mM, just enough to precipitate the chloride, the solubility of $AgIO_3$ found was 0.33 mM per liter. (4) When to (3) there was added KIO_3 in a concentration of 0.75 mM, the solubility of $AgIO_3$ decreased to 0.13 mM per liter. (5) By calculation from the solubility product found from (3) (Equation 7) the solubility of $AgIO_3$ in a solution containing 0.75 mM of

TABLE XV

Effect of Physical Solubility of $AgIO_3$ on Analysis of Low Chloride Urines by the Routine Iodate Method

Sample No.	Chloride concentration in original, undiluted urine, found by analysis		$AgIO_3$ solubility effect		$AgIO_3$ method values		
	By Van Slyke method	By $AgIO_3$ routine method	Found from (3) − (2)	From Curve A, Fig. 2	Corrected according to Fig. 2, (3) − (5)	Difference from Van Slyke values	
						(6) − (2)	(7) ÷ (2) × 100
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*per cent*
1	10.96	12.93	1.97	2.22	10.71	−0.25	−2.3
2	30.00	31.08	1.08	0.82	30.26	+0.26	+0.9
3	12.33	13.58	1.25	2.14	11.44	−0.89	−7.0
4	13.13	14.13	1.00	2.05	12.08	−1.05	−8.0
5	12.33	13.72	1.39	2.12	11.60	−0.73	−5.9
6	13.13	14.69	1.56	1.96	12.73	−0.40	−3.0
7	13.2	15.1	1.9	1.93	13.17	−0.03	−0.3
8	13.2	15.4	2.2	1.91	13.49	+0.29	+2.2
9	12.4	13.9	1.5	2.08	11.82	−0.58	−4.7
10	12.4	14.2	1.8	2.04	12.16	−0.24	−1.9
11	11.5	14.4	2.9	2.02	12.38	+0.88	+7.7
12	11.4	13.5	2.1	2.13	11.37	−0.03	−0.3
13	11.4	13.6	2.2	2.12	11.48	+0.08	+0.7
14	7.44	10.04	2.60	2.86	7.18	−0.26	−3.5
15	10.54	12.87	2.33	2.26	10.61	+0.07	+0.7
16	10.54	12.45	1.91	2.32	10.13	−0.41	−3.9
17	22.87	24.47	1.60	1.08	23.39	+0.52	+2.3
18	7.32	10.32	3.00	2.76	7.56	+0.24	+3.3
19	15.08	17.35	2.27	1.67	15.65	+0.57	+3.8
20	22.87	24.30	1.43	1.10	23.20	+0.33	+1.4
21	7.32	10.16	2.84	2.84	7.32	0.00	0.0
Average							±3.1

KIO_3 to begin with should be 0.125 mM per liter. The results of (2) and of (3) are in good agreement with this calculation.

When the result of the analysis of Sample 19 is calculated with the approximate $AgIO_3$ solubility of 0.12 mM per liter found above, the correction is 2.40 mM per liter of undiluted urine, and the final chloride calculated is 14.95 mM per liter compared with 15.08 mM per liter by the Van Slyke method. However, as has been shown above, the accuracy obtained with the use of the average solubility corrections of Fig. 2 will suffice.

When the concentration of chloride in the urine was increased by 100 mM per liter, by the addition of NaCl, gasometric analysis of the 20-fold diluted urine supernatant liquid gave 113.5 mM per liter of chloride or 98.6 per cent of the amount present (K = 0.990 for albuminous urines). This was further proof that neither the bile nor the protein was a disturbing factor when chloride was high enough to eliminate the possibility of any appreciable solubility of $AgIO_3$.

Serum[19]

Measurement of Amount of Iodate Dissolved per Mole of Chloride Present, When Serum Is Diluted with 0.085 M H_3PO_4 Solution and Shaken with $AgIO_3$—In Table XVI are summarized the results of analyses of 177 sera from normal, nephrotic, and nephritic individuals. For reaction with $AgIO_3$ the serum was diluted 10- to 25-fold with 0.085 M H_3PO_4, *without removal of the proteins*. Each serum or plasma was analyzed in duplicate, two portions of the diluted serum being shaken separately with $AgIO_3$. The total dissolved iodate was determined in some of the supernatant solutions by the gasometric method described in this paper, in others by the titrimetric method outlined in Paper II (Sendroy, 1937, *a*).

For comparison the chlorides were determined by triplicate analyses, according to the nitric acid digestion method of Van Slyke (1923–24).

The comparative results by the two methods are indicated in Table XVI by the ratio $100[IO_3]:[Cl]$. In this ratio $[IO_3]$ represents the moles of iodate per liter of serum found in solution after the reaction with $AgIO_3$,[20] while [Cl] represents moles of chloride per liter of serum found by the digestion method.

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[19] Part of the work presented in the following pages was done on serum, part on plasma. Since the results were identical with both, the term serum only will, for brevity, be used.

[20] Although theoretical considerations indicate a difference of 0.2 per

The average $[IO_3]:[Cl]$ ratio of 0.985 was obtained. There was no observable difference in either the average or of the variation of these K values between normal and pathological sera. The K value of 0.985 is incorporated in Table IV in the factors for calculation of gasometric serum chlorides. Consequently, in using these factors one automatically corrects for the fact that under the conditions of the analysis the ratio $[IO_3]:[Cl]$, or K, is 0.985 rather than 1.00.

Table XVI

Summary of Measurements of Ratio $[IO_3]:[Cl]$, in Analyses of Serum, without Removal of Proteins

Iodates were determined in serum diluted 10 to 25 times in 0.085 M H_3PO_4 and shaken with $AgIO_3$. Parallel chloride determinations, directly on serum, were made by Van Slyke's (1923-24) nitric acid digestion method. The ratio $100[IO_3]:[Cl]$ indicates moles of total dissolved iodate found per 100 moles of chloride found by the digestion method.

Species	No. of sera analyzed*	Ratio $\frac{100[IO_3]}{[Cl]}$			Procedure by which dissolved iodate was determined
		Average	Mean deviation from average	Extreme range	
Human	31	98.5	±0.41	97.5-99.3	Gasometric
	25	98.4	±0.59	97.4-99.6	Titrimetric
Horse	59	98.5	±0.47	97.5-99.6	Gasometric
	10	98.6	±0.30	98.1-99.2	Titrimetric
Ox	18	98.2	±0.35	97.6-99.1	Gasometric
	4	98.3	±0.20	98.1-98.5	Titrimetric
Sheep	10	98.1	±0.50	97.3-99.2	"
Dog	9	98.8	±0.39	98.2-99.3	Gasometric
Rabbit	2	98.2	0.00	97.8-97.8	"
	4	98.3	±0.20	98.1-98.5	Titrimetric
Mixed	5	98.6	±0.22	98.4-99.1	Gasometric
Total	124	98.5	±0.41	97.5-99.6	Gasometric
	53	98.4	±0.46	97.3-99.6	Titrimetric

* All analyses were in duplicate. The iodate determinations were made by shaking separate portions of serum or plasma with $AgIO_3$.

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cent between the results by 10-fold and by 25-fold dilution (Table VI), the difference is too small to be definitely demonstrable in serum analyses. All dilutions are, therefore, grouped together in Table XVI.

The accuracy of the value of 0.985 for K has been checked by other experiments in which the [Cl] factor of the ratio was determined simultaneously by both the nitric acid digestion and by the Sunderman-Williams (1933) alkaline digestion method (Table XVII). The two digestion methods were found to yield practically identical results with serum. As will be shown later (Tables XIX and XX), the accuracy of the ratio factor K = 0.985 has also been checked by experiments in which the [Cl] was fixed at a known value by adding known amounts of NaCl to serum which had been previously freed of chloride by dialysis.

TABLE XVII

Results of Comparison of Chloride Analyses in Plasma or Serum by Two Digestion Methods and by Gasometric and Titrimetric (Sendroy, 1937, a) Procedures of the Iodate Method

Each value given represents an individual plasma or serum sample.

Sample	Digestion methods, 3 cc. samples		Author's method, 0.5 cc. sample diluted to 10 cc. with 0.085 M H_3PO_4			
	Van Slyke (1923-24) analysis of [Cl]	Sunderman and Williams (1933) analysis of [Cl]	Gasometric procedure, 1 cc. supernatant, gas read at 0.5 cc. volume		Titrimetric procedure, 3 cc. supernatant, titrated with 0.03 N $Na_2S_2O_3$	
			Analysis of $[IO_3]$	Ratio* $\frac{100[IO_3]}{[Cl]}$	Analysis of $[IO_3]$	Ratio* $\frac{100[IO_3]}{[Cl]}$
	mM per l.	*mM per l.*	*mM per l.*		*mM per l.*	
Human plasma	106.0	106.5	104.8		103.9	
	106.0	106.2	104.7	98.7	104.0	98.0
Horse serum	93.0	93.0	91.8		91.6	
	93.5	93.3	92.2	98.7	91.7	98.4
Rabbit serum	102.7	102.8	101.2		101.0	
	103.2	103.2	100.9	98.3	101.0	98.2
Ox plasma	104.7	104.8	103.3		103.1	
	104.8	105.3	103.0	98.4	103.0	98.3

* The [Cl] used in the ratio is the mean of those found by the two digestion methods.

Reducing Effect of Dilute Serum on Dissolved Iodate from the Reaction of $AgIO_3$ with Chloride—As in the case of urine samples (Fig. 3), it was found that when chloride was precipitated with $AgIO_3$ from serum diluted with 0.085 M H_3PO_4, as the supernatant material was analyzed from time to time after centrifugation, there was a loss of iodate (Group A, Fig. 4). Although the values for $[IO_3]:[Cl]$ ratios found were lower for serum ($K = 0.985$) than for urine (nephritic, $K = 0.990$; normal, $K = 0.998$), the rate of disappearance of iodate after the first analysis was not so marked as for urine (Fig. 3). From the curves of Group A of Fig. 4 it is apparent that *if supernatant samples in the chloride analysis of serum are analyzed within at least 3 hours after centrifugation, no serious error from loss of iodate will occur.*

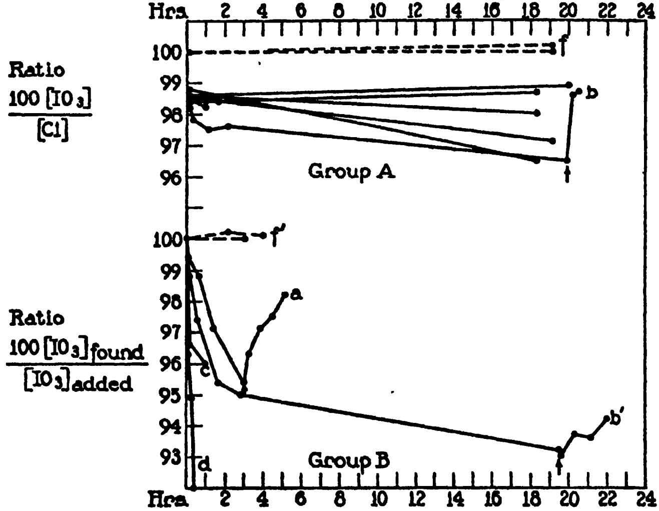

FIG. 4. Disappearance with time of dissolved iodate in diluted serum or deproteinized filtrates. Group A: iodate found from reaction of $AgIO_3$ and chloride. Supernatant fluid standing over the centrifuged solid AgCl and $AgIO_3$. Dotted lines, known chloride added to serum tungstic acid filtrates (samples *f*); solid lines, chloride precipitated from 0.085 M H_3PO_4 dilutions of serum. Sample *b* was dialyzed, chloride-free serum, with known chloride added. At the point indicated by the arrow, the tube of sample *b* was again shaken up and recentrifuged, with addition of a little more fresh $AgIO_3$. Group B: iodate found when added as KIO_3 or $KH(IO_3)_2$. Dotted lines, known iodate added to serum tungstic acid filtrates (samples *f'*); solid lines, known iodate added to 0.085 M H_3PO_4 dilutions of serum. Samples *a* and *b'* were 0.085 M H_3PO_4 dilutions of dialyzed, chloride-free sera. At the points indicated by arrows, $AgIO_3$ was added, and the samples were shaken up and centrifuged. Samples *c* and *d* were 0.085 M H_3PO_4 dilutions of normal sera.

Reducing Effect of Dilute Serum on Dissolved Iodate from Added KIO_3—From the fact that serum diluted with H_3PO_4 solution and shaken with $AgIO_3$ yielded on subsequent analysis only 0.985 mole of total dissolved iodate per mole of chloride originally present, instead of the 0.996 to 1.000 mole calculated theoretically or the 0.998 to 1.000 mole shown by pure NaCl solutions (Table XI), it appeared that a possible explanation was a slight immediate destruction of dissolved iodate by reducing agents in the serum. To test this possibility, KIO_3 in known amounts equivalent to the chloride found in normal serum was added to various samples of serum diluted with 0.085 M H_3PO_4. No $AgIO_3$ was added. The curves *c* and *d* of Group B (Fig. 4) show that the immediate destruction or loss of iodate in these sera was greater than when the iodate was derived from reaction of $AgIO_3$ with chloride. Curves *a* and *b′* were from dialyzed, chloride-free horse serum diluted with 0.085 M H_3PO_4 solution to 20 times the original serum volume. The initial concentration of IO_3 in these two samples, 5 mM, was approximately that of normal serum diluted 20-fold and shaken with $AgIO_3$ under the routine conditions of the author's method. The decrease in iodate in 3 hours, for samples *a* and *b′* amounted to about 5 per cent. For samples *c* and *d* the decrease was much greater than for *a* and *b′*, presumably on account of the presence of reducing substances which were dialyzed out of, and absent from samples *a* and *b′*.

Effect of $AgIO_3$ on the Partial Replacement of Lost Iodate—At the points marked by arrows in Fig. 4, $AgIO_3$ was added to samples *a* and *b′* to simulate (in the absence of AgCl, of course) the conditions in the usual serum chloride analysis. The mixture was shaken 2 minutes and centrifuged as in the routine analysis, and the iodate was determined again in the supernatant fluid. The fluid left after removal of the aliquot for analysis was mixed again with the same $AgIO_3$, shaken for 10 minutes, and analysis of the supernatant liquid was repeated. This procedure was again twice repeated, with shaking for 30 minutes.

Fig. 4 shows that this treatment restored the iodate of the supernatant solution of sample *a* to 98.2 per cent of that originally added. This is about the usual percentage of the theoretically expected iodate from chloride, found in acidified serum after reaction of its chloride with $AgIO_3$. Table VI shows that, with the concentration of KIO_3 present in solution, physical solution of

$AgIO_3$ could increase dissolved iodate by only 0.16 per cent of its concentration, whereas the increase in *a* was 2.8 per cent. The addition of $AgIO_3$ to sample *b′*, the iodate concentration of which had fallen to 93.0 per cent of the original after 19½ hours, caused an increase of only 1.2 per cent.

The same effect of $AgIO_3$ was demonstrated in the chloride analysis of sample *b* of Group A (Fig. 4). This dialyzed, chloride-free serum was the same as that of sample *b′* with known chloride, instead of iodate, added. After treatment with $AgIO_3$ and centrifugation, iodate analyses were made at intervals, as above. Upon being reshaken with the precipitated AgCl and $AgIO_3$ mixtures (time marked by arrow), after 20 hours the iodate was restored approximately to its original concentration.

When known iodate was added to Folin-Wu filtrates of serum, or when iodate was liberated from $AgIO_3$ by chloride in such filtrates, there was no destruction or loss of iodate, such as that found for undeproteinized serum.

Probable Reactions Which Result from Shaking Dilute Acidified Serum with Silver Iodate—From the results of Fig. 4 the following appears to be the possible sequence of events:

The chloride immediately reacts (to 99.6 to 99.8 per cent of completion) with $AgIO_3$, according to Equation 16. There are then present in the mixture solid $AgIO_3$ and AgCl, dissolved iodate and proteins, and other dissolved serum constituents which do not appear to influence the reactions considered.

There then begins a sharp reduction of the dissolved iodate to iodide. This reduction appears to be caused by the proteins, or by substances which are removable with them when the proteins are precipitated; for the disappearance of iodate does not occur in protein-free serum filtrate (samples *f* and *f′*).

However, the iodate reduced is partly replaced, because the iodide formed reacts at once with the excess $AgIO_3$ present, precipitating the I^- and dissolving IO_3^- which replaces that destroyed. The probable reactions are:

$$NaIO_3 + \text{protein (or other reductant)} \rightarrow NaI \tag{17}$$

$$NaI + AgIO_3 \rightarrow \downarrow AgI + NaIO_3 \tag{18}$$

If the reactions of Equations 17 and 18 were complete, the ratio $[IO_3]:[Cl]$ would be maintained at its theoretical level of 0.996

for 10-fold diluted serum. However, it appears either that not all of the destroyed iodate is reduced completely to iodide, or else that the reaction of Equation 18 is not complete; for the observed $[IO_3]:[Cl]$ ratio in 10- or 20-fold dilute serum is not 0.996, but only 0.985. Indeed, with reference to the reaction of Equation 18, it has already been noted that the ratio $[IO_3]:[\text{halide}]$ in 0.085 M H_3PO_4 solutions is always less when *iodide* is present than when all of the halide is chloride. Thus, when iodide was 5 per cent of the total halide (Table XIII), the ratio $[IO_3]:[I + Cl]$ was 0.985, of the same order of magnitude as that found for serum analyses.

The "protein" present in nephritic urine may cause the reactions of Equations 17 and 18 to occur in analyses of this material also, although to a less extent, because even in the most albuminous urines protein is not so concentrated as in serum. The $[IO_3]:[Cl]$ ratios observed in nephritic urines lie between the 0.985 met in serum analyses and the theoretical 0.998 obtained in analyses of salt solutions and normal urines.

Measurement of Iodate Dissolved per Mole of Serum Chloride, for Deproteinized Serum Filtrates Shaken with $AgIO_3$—Results with two types of serum filtrates are summarized in Table XVIII. In calculation of the $[IO_3]:[Cl]$ ratios the $[Cl]$ values used were the chloride concentrations per liter of serum obtained by nitric acid digestion of serum (Van Slyke, 1923–24). The $[IO_3]$ values used were the total dissolved iodate concentrations found per liter of serum in the analysis of 1:10 serum filtrates shaken with $AgIO_3$.

For the high $[IO_3]:[Cl]$ ratios averaging 1.017, thus obtained when the iodate method was applied to the tungstic acid filtrate (Folin and Wu, 1919), the probable explanation appears to be that the volume of fluid in the filtrate is really slightly less than the 10-fold serum volume assumed in calculating the $[IO_3]$ values. The 10-fold volume to which serum is diluted in the Folin-Wu tungstic acid precipitation includes not only the liquid phase, but also the solid matter of the precipitate. If the protein tungstate precipitate occupies 0.02 of the volume of the precipitate-fluid mixture, concentrations of serum crystalloids found by analysis of the *filtrate* would, when multiplied by 10 in the customary manner to estimate concentrations in serum, give values for the latter equal to 102 per cent of the correct figures. Any adsorption of a crystalloid by the precipitate would decrease the 2 per cent plus error. It appears probable from the results that the protein tungstate precipitate does not adsorb Cl^- ion, but that the zinc-protein precipitates formed in alkaline medium do adsorb it slightly. Analyses of the zinc filtrates indicate that the chloride in the zinc precipitation mixtures is approximately equally distributed, per volume unit, in the liquid and solid phases of the mixture, since the customary calculation of serum chloride values on this assumption gives correct results ($K = 0.995$).

An effect of volume displacement of fluid by protein precipitate, similar to that noted here in the tungstic acid precipitation was observed by Van Slyke, Hiller, and Berthelsen (1927) in a comparison of serum total base values, determined by ashing directly measured serum samples, with values determined by ashing 1:10 filtrates obtained by deproteinizing with trichloroacetic acid. The filtrates gave total base concentrations which, when multiplied by 10, were 3 to 4 per cent higher than those yielded by ashing directly measured serum. Similar effects of volume displacement on serum values calculated from trichloroacetic acid filtrate analyses have been reported by Ball and Sadusk (1936), who called attention to the lack of evidence for volume displacement "on other than trichloroacetic acid filtrates or in which sodium was not involved in the determination." As a matter of fact, careful inspection of Wilson and Ball's (1928) results reveals that in at least one-half of their comparisons of various methods the Whitehorn tungstic acid filtrates gave the highest results, up to a 1.5 per cent increase.

An effect of 2 per cent is more than one would estimate from the volume that would be occupied by serum protein in the dry form. This would be only about 5 per cent of the undiluted serum, or 0.5 per cent of the 10-fold diluted precipitation mixture. It appears that the precipitates as formed may hold bound or adsorbed water in amount equal to or greater than the weight of the dry proteins, and that this water may be withdrawn from the liquid phase, in so far as solvent action is concerned.

The results of Table XVIII were obtained after the possibility of effects, on analysis of deproteinized filtrates, of pH higher than those of 0.085 M H_3PO_4 solutions had been studied. Addition of phosphoric acid sufficient to make the pH 2.8 did not change the results obtained with 1:10 serum tungstic acid filtrates. On the other hand, when zinc filtrates were not acidified, the results were higher than those obtained by the digestion method. Acidification, as outlined, was therefore adopted as a preliminary procedure before the addition of $AgIO_3$ to zinc filtrates.

TABLE XVIII

Experiments with Protein-Free Serum Filtrates

Amounts of iodate dissolved by reaction of filtrates with $AgIO_3$ are compared with chloride determined directly in serum by the nitric acid digestion method of Van Slyke (1923–24).

Type of filtrate	Species of serum	No. of sera analysed	Ratio $\frac{100[IO_3]}{[Cl]}$			Procedure by which $[IO_3]$ was determined
			Average	Mean deviation from average	Extreme range	
Folin-Wu (1919), sodium tungstate + sulfuric acid (Van Slyke and Hawkins, 1928)	Horse	15	101.7	±0.50	101.0–102.4	Gasometric
		1	101.1			Titrimetric
	Human	16	102.0	±0.77	100.9–103.1	Gasometric
		3	102.2	±0.50	101.4–102.9	Titrimetric
	Ox	2	101.5			Gasometric
		1	101.6			Titrimetric
	Rabbit	1	101.7			Gasometric
		1	100.8			Titrimetric
	Total	34	101.7	±0.64	100.9–103.1	Gasometric
		6	101.7	±0.54	100.8–102.9	Titrimetric
Somogyi (1930), zinc sulfate + sodium hydroxide. Added H_3PO_4 to filtrate	Horse	1	99.5			Gasometric
		1	100.0			Titrimetric
	Human	1	99.3			"
	Ox	1	99.7			"
	Rabbit	1	98.9			"
	Total	5	99.5	±0.32	98.9–100.0	
Letonoff (1934), zinc borate. Added H_3PO_4 to filtrate	Ox	1	99.0			Titrimetric

Experiments in Which Known Amounts of NaCl or KIO_3 Were Dissolved in Dialyzed Serum—Serum protein solutions free from chloride were obtained by dialysis in collodion sacks against repeated changes of distilled water, until no test for chloride was obtained on a deproteinized sample of the serum within the sacks. The addition of a small amount of phosphoric acid to the dialyzed

TABLE XIX

Analyses of Dialyzed, Chloride-Free Sera, with Known Amounts of KIO_3 or NaCl Added, Analyzed with and without Deproteinization

Serum dilution to 10 or 11 times, concentration of added chloride or iodate, 10 or 9 mM per liter, in each case.

Analysis No.	Composition of sample analyzed	Value found for ratio $\frac{100[IO_3]\text{ found}}{[IO_3]\text{ added}}$ or $\frac{100[IO_3]\text{ found}}{[Cl]\text{ added}}$				Theoretical value for ratio
		Ox Serum I	Ox Serum II	Horse Serum III	Ox Serum IV	
	Serum diluted directly in 0.085 M H_3PO_4					
1	1 part 0.1 M KIO_3 + 9 parts 0.085 M H_3PO_4			100.0		100.0
2	1 part 0.1 M KIO_3 + 9 parts serum diluted 10 times in 0.085 M H_3PO_4	94.9*		97.8*	97.5*	100.0
3	1 part 0.1 M KIO_3 + 9 parts serum diluted 10 times in 0.085 M H_3PO_4 + $AgIO_3$	98.2		98.3		100.0
4	1 part 0.1 M NaCl + 9 parts serum diluted 10 times in 0.085 M H_3PO_4 + $AgIO_3$	97.7		98.7	97.7	99.6
	Tungstic acid filtrate of serum					
5	1 part 0.1 M KIO_3 + 9 parts filtrate of serum	100.1		100.0	99.9	100.0
6	1 part 0.1 M KIO_3 + 9 parts filtrate of serum + $AgIO_3$	100.4		100.0		100.0
7	1 part 0.1 M NaCl + 9 parts filtrate of serum + $AgIO_3$	99.9	100.2†	100.0	99.7	99.6
8	[1 part 0.1 M NaCl + 0.9 part serum] deproteinized with tungstic acid reagent mixture, diluted to 10 parts, + $AgIO_3$		101.5‡	101.7	101.4	99.6

* First analysis only. Successive subsequent analyses of the same supernatant fluid showed a decrease of from 2 to 4 per cent in each analysis.

† Chloride content, by Whitehorn (1920-21) titration, found to give value of ratio = 100.0.

‡ Chloride content, by Whitehorn (1920-21) titration, found to give value of ratio = 101.3.

TABLE XIX—*Concluded*

Analysis No.	Composition of sample analyzed	Value found for ratio $\frac{100[IO_3]\text{ found}}{[IO_3]\text{ added}}$ or $\frac{100[IO_3]\text{ found}}{[Cl]\text{ added}}$				Theoretical value for ratio
		Ox Serum I	Ox Serum II	Horse Serum III	Ox Serum IV	
	Zinc hydroxide filtrate of serum					
9	1 part 0.1 M KIO_3 + 9 parts filtrate of serum + 1 part 0.85 M H_3PO_4 + $AgIO_3$			100.0		100.0
10	1 part 0.1 M NaCl + 9 parts filtrate of serum + 1 part 0.85 M H_3PO_4 + $AgIO_3$			99.6		99.6
11	[1 part 0.1 M NaCl + 1 part serum] deproteinized with zinc sulfate and sodium hydroxide, diluted to 10 parts. 10 parts filtrate + 1 part 0.85 M H_3PO_4 + $AgIO_3$			100.5		99.6

material in the sack completely dissolved traces of precipitated protein. To the clear solution known amounts of KIO_3 or NaCl were added. The solutions were then either diluted with 0.085 M H_3PO_4 or were freed from protein by tungstic acid or zinc hydroxide precipitation. The resultant dilute solutions or filtrates were in some cases shaken with $AgIO_3$, in other cases not, as indicated in Table XIX. The dissolved iodate was determined in all. From the results in Table XIX the following conclusions may be drawn.

1. The $[IO_3]:[Cl]$ ratio of 0.985 in analyses of serum without protein removal, found when the [Cl] factor was ascertained by digestion analyses (Tables XVI and XVII), is confirmed by experiments in which the [Cl] factor was fixed by known additions of NaCl to dialyzed serum (Table XIX, Analysis 4).

2. The $[IO_3]:[Cl]$ ratios of 1.017 and 0.995 for tungstic acid and zinc hydroxide filtrates, respectively, found when serum [Cl] was determined by digestion analyses (Table XVIII), are approximated also when the chloride concentration is fixed by addition of known amounts of NaCl to dialyzed serum *before* precipitation of the proteins (Table XIX, Analyses 8[21] and 11). The probable

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[21] The results of the $AgIO_3$ method for chloride were confirmed in Analysis 8 (Ox Serum II) by determination of chloride according to the thiocyanate titration procedure of Whitehorn (1920–21).

rôles of fluid volume displacement and adsorption, by the protein precipitates, in affecting the $[IO_3]:[Cl]$ ratios have already been discussed in connection with Table XVIII.

3. KIO_3 added to tungstic acid filtrates and to zinc hydroxide filtrates of serum *after* precipitation of the proteins is completely recovered (Table XIX, Analyses 5, 6, and 9). On the contrary, when KIO_3 is added to serum which has merely been diluted with 0.085 M H_3PO_4, without removal of the proteins, some disappearance of dissolved iodate occurs (Table XIX, Analyses 2 and 3). These results indicate that the proteins, or material capable of precipitation with them, cause a gradual disappearance of dissolved iodate from dilute, acidified serum.

This behavior offers an explanation for the fact that, when such diluted serum is shaken with $AgIO_3$, the molar ratio of dissolved iodate to chloride is only 0.985 instead of the 0.996 calculated theoretically or the 0.998 found experimentally in experiments with protein-free chloride solutions. A part of the dissolved iodate is destroyed by reaction, presumably reduction, with the proteins or other colloids.

4. When known amounts of NaCl are added to filtrates of dialyzed serum *after* precipitation of the proteins (Analyses 7[22] and 10), and the filtrates are then shaken with $AgIO_3$, values of the $[IO_3]:[Cl]$ ratio approximating the theoretical 0.996 are obtained.

In these experiments there is no opportunity for protein precipitates to affect the results mechanically by volume displacement or adsorption, nor for dissolved proteins to destroy the dissolved iodate chemically by reduction. It appears that the only substances in serum causing the $[IO_3]:[Cl]$ ratio to deviate from theoretical are the proteins or material which is removed with the proteins when the latter are precipitated.

Non-Effect of Serum Dilution on the $[IO_3]:[Cl]$ *Ratio*—When serum was diluted with 0.085 M H_3PO_4 to from 10 to 30 times its volume, the variations in dilution caused little change in the yield of 0.985 mole of dissolved iodate per mole of chloride, as determined by digestion analysis. Tungstic acid filtrates, diluted with water to double volume after deproteinization also showed no change with dilution from the ratios (about 1.017) usually obtained in undiluted tungstic acid filtrates. From this it seems that the deviations of the $[IO_3]:[Cl]$ ratio from theoretical are unrelated to dilution *per se.* The ratio of concentrations of other serum constituents to concentration of chloride and of dissolved iodate was maintained constant in these experiments. Consequently, as dilution increased, the causative agent still remained present in the same proportion to chloride.

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[22] The results of the $AgIO_3$ method for chloride were confirmed in Analysis 7 (Ox Serum II) by determination of chloride according to the thiocyanate titration procedure of Whitehorn (1920–21).

In Table XX are the results of experiments in which the $[IO_3]:[Cl]$ ratio was determined under various conditions. In Series I are simple dilution experiments following the regularly prescribed technique for the author's method. In this series the ratio of chloride to other serum constituents remained constant, whether the dilution of serum was 10- or 30-fold. The tendency of this series was to give a fairly constant $[IO_3]:[Cl]$ ratio throughout the several dilutions.

In Series II the same serum samples were again diluted, but in such a manner that NaCl or KIO_3 was added to keep the *chloride* and hence the dissolved iodate *concentration constant.* Consequently, as dilution increased, the ratio of other serum constituents to chloride decreased. In this series the tendency was toward a decrease in deviation from the theoretical, as the dilution of serum was increased. Comparison of the same samples at the same dilutions of serum, of Series I and II, showed a decrease in deviation from theoretical as chloride or iodate was increased.

These results suggest that some constituent or constituents of serum depress the $[IO_3]:[Cl]$ ratio below the theoretical, and that the depression is proportional to the concentration of such constituents present per mole of chloride.

Effects of Various Factors on Serum Chloride Analyses by the Iodate Method. Temperature—At 15° and 35° the results of chloride analyses of serum were the same, showing that variations in room temperature, as in the case of salt solutions, have no significant effect.

Solubilities of Silver Iodate in Diluted Serum—In serum diluted to 10 times its volume with 0.085 M H_3PO_4 the solubility of $AgIO_3$ was the same as in 0.085 M H_3PO_4 alone. Hence, the fact that the $[IO_3]:[Cl]$ ratio is lower in analyses of dilute, protein-containing serum than in analyses of NaCl solutions is not caused by an effect of the proteins on $AgIO_3$ solubility.

Solubility of Air in Diluted Serum—Air solubility was found to

TABLE XX

Experiments to Determine Effect of Dilution in 0.085 M H_3PO_4 on Yield of Serum Chloride Analyses, without Deproteinization

Treatment and composition of sample	Ratio $\frac{100[IO_3]}{[Cl]}$ or $\frac{100[IO_3]}{[IO_3]\ added}$*			
Series I. Serum, and chloride or iodate, diluted together				
Original serum dilution in 0.085 M H_3PO_4	10	15	20	30
NaCl or KIO_3, approximate mM concentration in diluted serum	10.0	6.7	5.0	3.3
1. Mixed serum + $AgIO_3$	98.4		98.7	98.6
	−1.2		−1.0	−1.3
2. Dialyzed horse serum + NaCl + $AgIO_3$	98.5		97.9	98.1
	−1.1		−1.8	−1.8
3. Ox serum + $AgIO_3$	98.4	98.0		98.2
	−1.2	−1.7		−1.7
4. " " + KIO_3 (*no* $AgIO_3$)	96.8	96.7		97.2
	−3.2	−3.0		−2.7
Series II. Serum diluted, and chloride or iodate concentration kept *constant*				
Original serum dilution in 0.085 M H_3PO_4	10	15	20	30
NaCl or KIO_3, approximate mM concentration in diluted serum	10.0	10.0	10.0	10.0
1. Mixed serum + NaCl + $AgIO_3$	98.4		98.7	99.1
	−1.2		−0.9	−0.5
2. Dialyzed horse serum + NaCl + $AgIO_3$	98.5		98.5	98.5
	−1.1		−1.1	−1.1
3. Ox serum + NaCl + $AgIO_3$	98.4	98.6		99.0
	−1.2	−1.0		−0.6
4. " " + KIO_3 (*no* $AgIO_3$)	96.8	98.5		99.1
	−3.2	−1.5		−0.9

* Deviation from the theoretical for each dilution, as given in Table VI, is indicated directly below the ratio value. The values of [Cl] in the sera were determined by the Van Slyke digestion method, and are the values of [Cl] in the $[IO_3]:[Cl]$ ratios in Series I, Samples 1 and 3. In Series I and Series II, Sample 2, [Cl] values in the ratio $[IO_3]:[Cl]$ are those of NaCl added to dialyzed serum. In Series II, Samples 1 and 3, values of [Cl] are calculated from serum chloride + added NaCl. In Series I and Series II, Sample 4, values of $[IO_3]$ added are calculated from KIO_3 added to serum. In all analyses, values of $[IO_3]$ are from determined gasometric iodate measurements.

be the same, within the limits of error of analysis, in serum diluted with 0.085 M H_3PO_4, and in 0.085 M H_3PO_4 alone. Hence, the use of 0.085 M H_3PO_4 for gasometric blank analyses involves no error.

Non-Effect of Lipid—Sera from patients with marked lipemia gave $[IO_3]:[Cl]$ ratios (determined as in the experiments of Table XVI) like those of normal sera. The likelihood that fats affect the yields of iodate from chloride in serum was further diminished by the results of the addition of 5 per cent by volume of olive oil to sodium chloride solutions. Analysis of the thoroughly mixed suspension showed that the oil was entirely inert, with regard to both the solubility of solid silver iodate and the amount of iodate liberated by reaction with chloride. The theoretical amount of iodate, from the reaction of $AgIO_3$ and chloride, was found in the separated water phase.

Effect of Hemoglobin on the $[IO_3]:[Cl]$ Ratio—The presence in serum of red blood cells, intact or laked, in amounts up to 5 cc. of cells per 100 cc. of serum has no effect on the results of chloride analyses carried out by the iodate method without deproteinization. The chloride values found agreed exactly with those by the Van Slyke digestion method, when the K factors of serum were used in calculating the results of the iodate method. Cells do have a slight reducing effect on iodate, so that the cell concentration in ordinary whole blood diminishes the ratio $[IO_3]:[Cl]$ by about 1 per cent below the ratio found for serum. Amounts of cell material that may enter serum from accidental hemolysis of blood samples, however, are not sufficient to affect chloride results by the iodate method.

Comparison of Ultramicro- and Microprocedures with the Macroprocedures for Serum Chlorides—In Table XXI are given the results of comparative analyses of serum for very small samples, 0.02 cc. to 0.2 cc., and larger 1.0 cc. samples, all analyzed directly, without deproteinization, by the iodate method. Each analysis was carried out in duplicate, by shaking separate portions of serum with $AgIO_3$. Analyses of whole blood by deproteinization have yielded results of the same order of constancy as those given in Table XVIII. It is apparent that very little sacrifice in accuracy is entailed by the use of ultramicro and micro samples, when analyzed according to the technique given in this paper.

Claudius (1922), Patterson (1928), and Rappaport (1933) have given various modifications of the "open Carius" titrimetric method applied to small samples. Claudius used 0.02 cc. samples, but gave no data for serum analyses. Patterson used 0.2 cc. with an indicated accuracy of about ±1 per cent for serum samples. Rappaport used 0.1 cc. samples and reported results of about the same order of accuracy in the analysis of five samples. So far as

TABLE XXI

Comparison of Chlorides Determined in Serum by the Macro, Micro, and Ultramicro Forms of the Iodate Method

Sample No.	Material	Macroanalysis				Micro- or ultramicroanalysis				Deviation of micro from macro results
		Sample volume	Dilution with 0.085 M H_3PO_4*	Method†	Chloride found	Sample volume	Dilution with 0.085 M H_3PO_4	Method†	Chloride found	
		cc.	*cc.*		*mM per l.*	*cc.*	*cc.*		*mM per l.*	*mM per l.*
1	Human plasma	1	+30	G.	104.1	0.02	+0.6	G.	104.4	+0.3
2	Ox serum	1	+30	"	103.5					
				T.	103.8	0.02	+0.6	T.	103.3	−0.3
3	" "	1	+30	"	103.8	0.02	+0.6	"	105.1	+1.3
4	Horse serum	0.5	To 10	G.	91.8					
				T.	91.3	0.02	+0.6	"	92.5	+0.9
5	Ox serum	1	" 30	G.	97.9					
		1	+30	"	98.4	0.05	+1.5	G.	98.5	+0.3
6	" "	1	+30	"	98.9	0.05	+1.5	"	98.4	−0.5
7	Human plasma	1	To 25	"	109.8	0.1	+2.0	"	109.2	−0.6
8	" "	1	" 10	"	103.3	0.1	+2.0	"	103.8	+0.5
		1	" 25	"	103.9	0.2	+5.0	"	103.5	−0.4
9	" "	1	" 25	"	107.5	0.2	+5.0	"	107.6	+0.1
10	" "	1	" 10	"	107.5	0.2	+5.0	"	107.6	0.0
		1	" 25	"	107.7					
11	" "	1	+10	"	105.9	0.2	+2.0	"	106.3	+0.4
						0.2	+5.0	"	107.1	+1.2
12	" "	1	To 25	"	106.6	0.2	+5.0	"	107.0	+0.4
13	" "	1	" 25	"	89.1	0.2	+5.0	"	89.3	+0.2
14	" "	1	" 25	"	102.1	0.2	+5.0	"	102.1	0.0

* By "+30" is meant that 1 volume of serum was mixed with 30 volumes of 0.085 M H_3PO_4. "To 30" indicates dilution of 1 volume of serum to 30 volumes with the H_3PO_4.

† G. = gasometric analysis of present paper; T. = titrimetric analysis of following paper (Sendroy, 1937, *a*).

the author is aware, the present method for plasma chloride determinations is the only one available for which there has been demonstrated the degree of accuracy of the results of Table XXI for samples of 0.02 cc. and 0.05 cc.

Whole Blood

Results with Protein-Free Filtrates[23]—As for serum, the results were gaged for accuracy by comparison with results obtained by the Van Slyke (1923–24) digestion method and by the Sunderman and Williams (1933) procedure. The latter was accepted as standard; it gave whole blood chloride values averaging 0.5 per cent higher than the Van Slyke simple nitric acid digestion method. Table XXII shows that *whole blood tungstic acid filtrates* (not

TABLE XXII

Summary of Results of Comparative Chloride Analyses in Human Whole Blood by the Sunderman and Williams (1933) Digestion Method, and by the Iodate Method Applied to Deproteinized Samples

Procedure		No. of analyses	Average		Extreme range of ratio values
Sample treatment	Measurement of iodate		Values for ratio $\frac{100[IO_3]}{[Cl]}$	Deviation from mean of average ratio values	
Filtrate of (tungstic acid + H_3PO_4) diluted to 25 times serum volume	Gasometric Titrimetric	22 20	100.0 100.0	±0.74 ±0.58	98.7–101.3 98.8–101.2
Filtrate of zinc hydroxide (1:10) diluted to 1.1 times volume with H_3PO_4	Gasometric Titrimetric	2 2	98.9 98.7		98.2– 99.6 98.0– 99.3

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[23] When diluted cell suspension or whole blood containing the cell colloids is shaken with $AgIO_3$, the supernatant fluid contains material which reduces the dissolved iodate, so that the iodate subsequently measured is less than theoretically corresponds to the chloride present. With whole blood diluted 1:20 in 0.085 M H_3PO_4 the effect is constant enough to permit use of a correction factor (K = approximately 0.975) without much loss of accuracy but it is preferable to remove the proteins. With separated cells, removal of the proteins is necessary.

Folin-Wu preparations, but those prescribed for this method) gave [IO_3]: [Cl] ratios approaching the theoretical value of 0.999 (for these concentrations of chloride) in two series, gasometric and titrimetric (Sendroy, 1937, *a*), of twenty different samples each. Tungstic acid *serum* filtrates diluted 1:10, have been shown to yield 1.017 moles of iodate per mole of serum chloride. The lower yield of these whole blood filtrates, diluted 1:25, is probably attributable to the greater reducing action of cell constituents other than proteins on iodate—an effect which approximately balances the positive "volume displacement" error.

Somogyi zinc hydroxide filtrates of whole blood were analyzed (Table XXII). While serum results (Table XVIII) indicated values close to the theoretical [IO_3]: [Cl] ratio of 0.996 (for these concentrations of chloride), the yields for whole blood filtrates, prepared in the same dilutions as serum filtrates, were slightly lower, averaging 0.988. Because of the scarcity of these data, the slight discrepancy in serum and whole blood filtrate values cannot be stated to be significant.

SUMMARY

In the proposed method chloride-containing solutions are shaken with silver iodate, whereby the Cl^- in solution is replaced by an equimolar amount of IO_3^-. The IO_3^- is then determined by its oxidative reaction with alkaline hydrazine, $2IO_3^- + 3N_2H_4 = \uparrow 3N_2 + 6H_2O + 2I^-$, with manometric measurement of the N_2 in the Van Slyke-Neill apparatus. Since IO_3^- as an oxidizing agent is hexavalent, 1 equivalent of Cl^- is represented by 6 equivalents of oxidized hydrazine. This multiplication gives the iodate method a unique advantage for microanalyses: it is possible to determine, with an error not exceeding 1 per cent, the chloride in as little as 0.02 cc. of serum. Moreover, the measurements are directly proportional to the amount of chloride present.

The method is rapid; only 2 minutes shaking are required for the reaction between $AgIO_3$ and Cl^-, while the reaction with hydrazine is instantaneous. The procedure also has the advantages of the manometric technique in freedom from standard solutions and in speed and accuracy of micromeasurement. No removal of proteins, either by precipitation or digestion, from urine, or plasma or serum, is required, although protein-free filtrates of serum or of whole blood may also be used for analysis.

The author is indebted to Dr. D. D. Van Slyke for his helpful criticism, and to Dennis Kertesz, who did the major portion of the analytical work of this paper.

BIBLIOGRAPHY

Ball, E. G., and Sadusk, J. F., Jr., *J. Biol. Chem.*, **113**, 661 (1936).

Brown, A. S., and MacInnes, D. A., *J. Am. Chem. Soc.*, **57**, 489 (1935).

Claudius, D., *Verhandl. 34 Kong. deutsch. Ges. inn. Med.*, Wiesbaden, 138 (1922).

Eisenman, A. J., *J. Biol. Chem.*, **82**, 411 (1929).

Folin, O., and Wu, H., *J. Biol. Chem.*, **38**, 81 (1919).

Harvey, S. C., *Arch. Int. Med.*, **6**, 12 (1910).

Haslewood, S. A. D., and King, E. J., *Biochem. J.*, **30**, 903 (1936).

Isaacs, M. L., *J. Biol. Chem.*, **53**, 17 (1922).

Landolt-Börnstein-Roth physikalish-chemische Tabellen, Berlin, 4th edition, 456 (1912).

Letonoff, T. V., *J. Lab. and Clin. Med.*, **20**, 1293 (1934).

Patterson, J., *Biochem. J.*, **22**, 788 (1928).

Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore (1932).

Rappaport, F., *Klin. Woch.*, **12**, 1774 (1933).

Riegler, E., *Z. anal. Chem.*, **40**, 633 (1901); **41**, 17 (1902).

Sendroy, J., Jr., *Proc. Am. Soc. Biol. Chem.*, **8**, lxxxi (1935) (*J. Biol. Chem.*, **109** (1935)); *J. Biol. Chem.*, **120**, 405 (1937, *a*); **120**, 419 (1937, *b*); **120**, 441 (1937, *c*).

Sendroy, J., Jr., Seelig, S., and Van Slyke, D. D., *J. Biol. Chem.*, **106**, 463 (1934).

Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).

Sunderman, F. W., and Williams, P., *J. Biol. Chem.*, **102**, 279 (1933).

Van Slyke, D. D., *J. Biol. Chem.*, **58**, 523 (1923-24); **73**, 121 (1927).

Van Slyke, D. D., and Hawkins, J. A., *J. Biol. Chem.*, **79**, 739 (1928).

Van Slyke, D. D., Hiller, A., and Berthelsen, K. C., *J. Biol. Chem.*, **74**, 659 (1927).

Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, **61**, 523 (1924).

Westfall, B. B., Findley, T., and Richards, A. N., *J. Biol. Chem.*, **107**, 661 (1934).

Whitehorn, J. C., *J. Biol. Chem.*, **45**, 449 (1920-21).

Wilson, D. W., and Ball, E. G., *J. Biol. Chem.*, **79**, 221 (1928).

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MICRODETERMINATION OF CHLORIDE IN BIOLOGICAL FLUIDS, WITH SOLID SILVER IODATE

II. TITRIMETRIC ANALYSIS

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(Received for publication, May 8, 1937)

In Paper I of this series (Sendroy, 1937, *a*) a system of chloride analysis based on the reaction

$$(1)\qquad \underset{\text{soluble}}{NaCl} + \underset{\text{insoluble}}{AgIO_3} \rightleftarrows \underset{\text{insoluble}}{AgCl} + \underset{\text{soluble}}{NaIO_3}$$

was outlined in detail, with gasometric measurement of the iodate resulting from the reaction. In this paper titrimetric measurement of the iodate is described.

Titrimetric measurement of the iodate is directly applicable to the supernatant fluid in the $AgIO_3$ precipitation of chloride from *salt solutions*, *urine*, *serum*, and *protein-free filtrate* of serum and of whole blood.[1] For the titrimetric analysis of *blood* only protein-free filtrates may be used.

The measurement is carried out according to the classical reaction for iodate in acid solution in the presence of an excess of potassium iodide. The equation applicable to the present measurements is

$$(2)\qquad KIO_3 + 5KI + 6H_3PO_4 = 3I_2 + 3H_2O + 6KH_2PO_4$$

The iodine evolved from the reaction of the iodate with acidified KI in excess is titrated against sodium thiosulfate with starch as indicator. Since 6 equivalents of iodine result from 1 of chloride, this reaction is 6 times as sensitive as the one on which the iodometric titration procedure of McLean and Van Slyke (1915), or that of Conway (1935), is based.[3]

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[1] The term "serum" is used here, as in the previous paper, for both plasma and serum.

What has been said of the advantages of the method in its gasometric form applies equally well to the titrimetric form, except that all titrimetric measurements must be compared against a known, standard iodate solution. In accuracy, convenience, and rapidity, the method in its titrimetric form is little different from the gasometric. Here also, samples of serum as small as 0.02 cc. may be used, with no loss of accuracy. By the reaction of Equation 2 about 3.2 cc. of 0.003 N $Na_2S_2O_3$ are required for titration of the iodine in the supernatant fluid available from such a sample of serum. The sensitivity of the starch-iodine end-point makes titrations easily possible to within 0.01 cc.

DESCRIPTIVE

Reagents—

Approximately 0.85 M, 0.34 M, 0.17 M, and 0.085 M Phosphoric Acid Solutions.

M/15 Potassium Acid Phosphate Solution.

Caprylic Alcohol.

Silver Iodate, Powder, C.P.

Tungstic Acid Reagent (Folin-Wu, 1919).

Zinc Hydroxide Reagent (Somogyi, 1930).

The preparation of the above reagents is described in the corresponding section of Paper I (Sendroy, 1937, *a*). In addition, there are the following:

5 Per Cent Potassium Iodide Solution—This is freshly prepared every day. 5 gm. of KI are dissolved in 100 cc. of water.

2 Per Cent Starch Solution—Of Lintner's soluble starch, 2 gm. are ground into suspension with a little water, then boiled in 100 cc. of saturated NaCl solution for 5 or 10 minutes. This reagent keeps indefinitely.

0.5 Per Cent Starch Solution—The 2 per cent solution is diluted 4 times with saturated NaCl solution.

Approximately 0.12 M (= 0.12 N) Sodium Thiosulfate Solution—About 30 gm. of $Na_2S_2O_3 \cdot 5H_2O$ are diluted to 1 liter with water.

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[3] As far as the writer is aware, there has been no previous application of this reaction to the analysis of chlorides. See the foot-note to the title page of Paper I (Sendroy, 1937, *a*).

This stock solution and the weaker solutions made from it by simple dilution are kept in the dark, in amber bottles. If aged properly, they will become very stable. Without any precautions against CO_2 we have kept even 0.001 N solution unchanged for 5 days.

Approximately 0.03 N, 0.0075 N, and 0.003 N sodium thiosulfate solutions are made from the 0.12 N solution by approximate dilution with water, to 4, 16, and 40 times volume, respectively.

0.05 M (= 0.6 N) Standard Potassium Biiodate Solution—Exactly 19.498 gm. of $KH(IO_3)_2$ are dissolved in water to make 1 liter of solution. This standard stock solution keeps indefinitely.

Standard 0.03 N and 0.0075 N potassium biiodate solutions are made from the 0.6 N solution by dilution with *0.085 M H_3PO_4 solution* to 20 and 80 times volume, respectively.

Procedure

The general procedure with titrimetric analysis of the iodate consists of the same steps described in the "Procedure" of Paper I. For the treatment of samples up to the point where the solid $AgIO_3$ and AgCl are separated from the supernatant fluid, the reader is referred to the details in the corresponding section of that paper (Sendroy, 1937, *a*) from steps (1) through (4) to the beginning of the description of the "Gasometric determination of total dissolved iodate."

Titrimetric Measurement

As in the gasometric measurement, the volume of the supernatant sample analyzed will depend on the size of the initial sample of original material and the extent of the dilution. Supernatant samples of from 0.5 cc. to 3.0 cc. volume are pipetted into a small test-tube (15 × 125 mm.).[3] 0.085 M H_3PO_4 is added to bring the volume to 3.0 cc. or 1.5 cc. Freshly prepared 5 per cent potassium iodide solution, in suitable amounts, is added and allowed to react for at least 30 seconds. The liberated iodine is then titrated with sodium thiosulfate solution. When the yellow color has been almost completely discharged, the starch indicator solution is added and the titration is carried on to an almost completely colorless, permanent end-point. The use of a constant light source, such as a daylight lamp, is desirable for this titration.

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[3] Wherever the use of a cotton plug is indicated for withdrawal of samples of the supernatant fluid in the gasometric procedure, such a filter should be used in the corresponding procedure of the titrimetric analysis.

The thiosulfate solution used is in every case of a strength such that from 2.5 to 3.2 cc. are required for complete titration of the sample. The thiosulfate is delivered, drop by drop, from a Bang 3 cc. microburette (Peters and Van Slyke (1932) p. 13) calibrated in 0.01 cc. or 0.02 cc. intervals and tipped with a pointed capillary delivering 100 to 150 drops per cc. Increments of reagent of the order of magnitude of 0.002 cc. may thus be accurately delivered.

The *standardization* of the thiosulfate is carried out by titration of an equivalent amount of the standard iodate solution in place of the supernatant sample, in *exactly* the same way as described above. No blank analysis is made in the titrimetric procedure. It is necessary only to ascertain that the acid solution used to dilute the original sample is chloride-free, and that it develops no blue color with starch when potassium iodide is added to it.

In the analysis of urine samples the yellow color of the original sample does not interfere with the preliminary iodine titration before the addition of starch. The extent of dilution with phosphoric acid, for dilute or for concentrated samples, is sufficient to produce an almost colorless solution. In the analysis of serum samples the same holds true. Upon the addition of the KI to the supernatant fluid of undeproteinized serum samples, the iodine will be observed to separate out, as if attached to protein particles. As the titration proceeds, however, this clears up, and the solution finally becomes almost entirely clear and transparent.

Specific directions for chloride determination in various kinds of materials, with titrimetric analysis of the iodate, are given below. For details of the steps leading up to the actual titrimetric analysis of the supernatant samples, the reader is referred to the corresponding sections in Paper I (Sendroy, 1937, *a*).

A. Determination of Chloride in Inorganic Material—Samples are diluted as described in the corresponding Section A of Paper I. Approximately 0.1 M neutral salt solutions or 0.1 N HCl solutions are diluted 10 to 30 times with 0.085 M H_3PO_4, then analyzed as described for serum analyses below, except that no caprylic alcohol is necessary.

B. Determination of Chloride in Urine—As for gasometric analysis, samples may be analyzed according to (*a*) a routine method used for all samples, or (*b*) a more accurate method.

Routine Method—The urine is diluted 20-fold with 0.17 M H_3PO_4, as described in the corresponding Section B of Paper I. Samples of 0.5 cc. (from very concentrated, normal urines) to 3.0 cc. (from low chloride urines) of the supernatant fluid are taken, and 0.085 M H_3PO_4 is added when needed to make the volume 3.0 cc. The analysis is completed as outlined in the previous section headed "Titrimetric measurement" by the addition of 1.0 cc. of 5 per cent KI, 1 drop of 2 per cent starch solution, and 0.03 N $Na_2S_2O_3$ delivered from a 3 cc. microburette. For the standardization, 2 cc. of 0.03 N $KH(IO_3)_2$ plus 1 cc. of 0.085 M H_3PO_4 are titrated with 0.03 N $Na_2S_2O_3$ in exactly the same way.

The remarks made in Paper I concerning the accuracy of the routine method for urine apply equally well to the titrimetric analysis. The correction for $AgIO_3$ solubility in the case of low chloride samples is also used for titrimetric analyses. However, the use of thiosulfate of a single strength (0.03 N) for all samples necessarily limits the accuracy of the measurements in the case of low chloride urines. The resulting errors are, nevertheless, much less than those obtained by the usual Volhard titration by difference (Sendroy, 1937, *b*), especially when protein is present. For analysis by 20-fold dilution the titration with 0.03 N $Na_2S_2O_3$ will range from 0.3 cc. for a 3 cc. sample of the supernatant fluid from a urine 10 mM in chloride to about 2.4 cc. for a 1 cc. sample of supernatant fluid from a urine 240 mM in chloride.

Precise Method—Dilution with 0.17 M H_3PO_4 is made as described in the corresponding Section B of Paper I (Sendroy (1937, *a*) Table I). Samples of 2 cc. of the supernatant fluid are diluted with 1 cc. of 0.085 M H_3PO_4 and 1 cc. of 5 per cent KI solution. Titration is carried out with 0.03 N $Na_2S_2O_3$, 1 drop of 2 per cent starch being added near the end-point. Standardization is carried out as for the *routine* procedure.

C. Determination of Chloride in Plasma or Serum, without Deproteinization—The method of preparing samples, from 1.00 cc. down to 0.02 cc., by dilution with 0.085 M H_3PO_4, is the same as that described in the corresponding Section C for the gasometric determination (Sendroy, 1937, *a*). The use of samples of various volumes and titration with $Na_2S_2O_3$ of different, suitable concentrations make possible the maintenance of a high degree of accuracy even for 0.02 cc. samples (Sendroy (1937, *a*) Table XXI).

Macrodetermination—A sample of 0.5 cc. of serum is diluted 20 times and prepared for iodate analysis, as in the gasometric method. Of the supernatant fluid, 3 cc. are taken, usually without the necessity of a cotton plug in the pipette. Then 1 cc. of 5 per cent KI is added, and the titration is carried out with 0.03 N $Na_2S_2O_3$, 1 drop of 2 per cent starch being added near the end-point. For the thiosulfate standardization 2 cc. of 0.03 N $KH(IO_3)_2$ plus 1 cc. of 0.085 M H_3PO_4 plus 1 cc. of 5 per cent KI are titrated with 0.03 N $Na_2S_2O_3$, 1 drop of 2 per cent starch being added near the end-point.

Microdetermination—Samples of 0.2 cc. or 0.1 cc. of serum are diluted and prepared for iodate analysis, as in the gasometric method. Of the supernatant fluid, samples of 1 cc. are taken with a pipette equipped with a cotton plug. Then 2 cc. of 0.085 M H_3PO_4 and 0.3 cc. of 5 per cent KI are added. The titration is carried out with 0.0075 N $Na_2S_2O_3$, 1 drop of 0.5 per cent starch solution being added near the end-point. For the thiosulfate standardization 2 cc. of 0.0075 N $KH(IO_3)_2$ plus 1 cc. of 0.085 M H_3PO_4 plus 0.3 cc. of 5 per cent KI are used.

Ultramicrodetermination—Samples of 0.05 cc. or 0.02 cc. are diluted and prepared for iodate analysis, as in the gasometric method. *For the 0.05 cc. analysis*, the titration of a 1 cc. sample of the supernatant fluid is carried out exactly as above for *micro* samples.

For the 0.02 cc. analysis, the sample of supernatant fluid is withdrawn in a 0.5 cc. pipette, calibrated between marks. A cotton plug filter is used in the pipette. To the sample are added 1.0 cc. of 0.085 M H_3PO_4 and 0.1 cc. of 5 per cent KI. The titration is carried out with 0.003 N $Na_2S_2O_3$, 1 drop of 0.2 per cent starch solution being added near the end-point. For the thiosulfate standardization 1 cc. of 0.0075 N $KH(IO_3)_2$ plus 1 cc. of 0.085 M H_3PO_4 plus 0.1 cc. of 5 per cent KI are used.

D. Determination of Chloride in Protein-Free Filtrates of Plasma, Serum, or of Whole Blood—These filtrates are prepared for analysis as described in the corresponding Section D (Sendroy, 1937, *a*) on gasometric analysis.

(*a*) *Tungstic acid filtrates of plasma or serum.* When enough filtrate is available, samples of 1.5 cc. or 1.0 cc. of the supernatant fluid are taken in a pipette without a cotton plug. There are then added 1.5 or 2.0 cc. of 0.085 M H_3PO_4, respectively, followed by 1.0 cc. of 5 per cent KI. Titration is carried out with 0.03 N $Na_2S_2O_3$ and 1 drop of 2 per cent starch solution. Standardization is carried out with 2 cc. of 0.03 N $KH(IO_3)_2$, as described above for the *macro*determination without deproteinization.

If there is a scarcity of filtrate, samples of 0.5 cc. of the supernatant fluid, taken in a pipette equipped with a cotton plug, may be used. There are added 1.5 cc. of 0.085 M H_3PO_4, 0.3 cc. of 5 per cent KI, and 0.0075 N $Na_2S_2O_3$. Near the end-point 1 drop of 0.5 per cent starch is added. Standardization is carried out with 2 cc. of 0.0075 N $KH(IO_3)_2$, as described above for the *micro*determination without deproteinization.

(*b*) *Tungstic acid filtrates of whole blood* are made, and prepared for analysis as described for the gasometric method. The usual Folin-Wu filtrate may not be used. Of the supernatant fluid from the *macro* (1 cc.) or the *micro* (0.2 cc.) determination, 1 cc. samples are taken. There are added 2.0 cc. of 0.085 M H_3PO_4 and 0.3 cc. of 5 per cent KI. Titration is carried out with 0.0075 N $Na_2S_2O_3$, standardized as described above for the *micro*determination without deproteinization.

(*c*) *Zinc hydroxide filtrates of plasma or serum* are made, and prepared for analysis as described for the gasometric method. Samples of 1.5, 1.0, or 0.5 cc. of the supernatant fluid are analyzed as described above (*a*) for tungstic acid filtrates of serum.

Calculations

From theoretical considerations (Sendroy, 1937, *a*) and experimental results, 1 mole of iodate found by analysis corresponds to $1/K$ mole of chloride originally present. In the titration 1 mole of iodate is equivalent to 6 moles of thiosulfate. Hence, the general equation for the calculation of titrimetric results is:

$$[\mathrm{Cl}]_t = v \times \frac{n \times d \times 1000}{6 \times K \times s} \qquad (3)$$

where

$[\mathrm{Cl}]_t$ = mM chloride per liter of original sample

v = cc. volume of thiosulfate used for titration of the supernatant sample

TABLE I

Factors for Calculation of Titrimetric Chloride Analyses by Iodate Method

The chloride concentration of the original samples is found by multiplying the product (titration volume, v, × standardized normality, n) of the $Na_2S_2O_3$ by the factor f, according to Equation 4, $[Cl] = v \times n \times f$.

Material	Analysis	Original sample volume	d = dilution factor of original sample in supernatant liquid	s = volume of supernatant liquid analyzed	Approximate normality of $Na_2S_2O_3$ used	K = ratio $\frac{[IO_3]}{[Cl]}$	f = factor by which to multiply (titration volume, v, × standardized normality, n) of the $Na_2S_2O_3$, to obtain		
							mM Cl per liter	Gm. NaCl per liter	Mg. NaCl per 100 cc.
		cc.		cc.					
Normal urine	Routine	0.5	20	0.5	0.03	0.998	6,680*	390.8*	
				1.0	0.03	0.998	3,340	195.4	
				3.0	0.03	0.998	1,113	65.1	
Albuminous urine	Routine	0.5	20	0.5	0.03	0.990	6,734*	393.9*	
				1.0	0.03	0.990	3,367	197.0	
				3.0	0.03	0.990	1,122	65.6	
Serum, without deproteinization	Macro	0.5	20	3.0	0.03	0.985	1,128		6,599
	Micro	0.2	26.05	1.0	0.0075	0.985	4,408		25,787
		0.1	31.05	1.0	0.0075	0.985	5,254		30,736
	Ultramicro	0.05	31.05	1.0	0.0075	0.985	5,254		30,736
		0.02	31.15	0.5	0.003	0.985	10,540		61,659
Serum, tungstic acid filtrate			10	1.5	0.03	1.017	1,093		6,394
			10	1.0	0.03	1.017	1,639		9,588
			10	0.5	0.0075	1.017	3,278		19,176
Whole blood, tungstic acid filtrate	Macro	1.0	25	1.0	0.0075	1.000	4,167		24,377
	Micro	0.2	25	1.0	0.0075	1.000	4,167		24,377

Serum, zinc hydroxide filtrate			11†	1.5	0.03	0.995	1,228		7,184
			11†	1.0	0.03	0.995	1,843		10,782
			11†	0.5	0.0075	0.995	3,685		21,557

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* *If urine analyzed by the routine method is calculated to contain less than 60 mM of chloride per liter (or 3.51 gm. of NaCl per liter), correction for $AgIO_3$ solubility must be made according to Fig. 2 of the previous paper (Sendroy (1937, a) p. 363).*

† If the serum filtrate + H_3PO_4 mixture is prepared by diluting 9 volumes of filtrate to 10 volumes (see foot-note 13 (Sendroy, 1937, *a*)), $d = 11.11$ and the factors f are multiplied by 1.010.

n = standardized normality of the thiosulfate

d = dilution factor for the number of times original sample was diluted, with H_3PO_4 or protein precipitant, before shaking with $AgIO_3$

s = cc. volume of supernatant sample used for titration

$K = \frac{[IO_3]}{[Cl]}$ = empirical reaction yield factor expressing the ratio of the concentration of iodate, found by analysis, to the concentration of chloride originally present, per liter of original sample

For convenience in calculating, Equation 3 can take the form

$$[Cl] = v \times n \times \left(\frac{d \times 1000}{6 \times K \times s}\right) = v \times n \times f \qquad (4)$$

When results in terms of gm. of NaCl per liter, or mg. of NaCl per 100 cc. are desired, the values of f for mM of Cl per liter are multiplied by 0.0585, or 5.85, respectively.

In Table I are given values for the factor f, under different conditions of analysis. When the product (titration volume, v, × standardized normality, n) of the $Na_2S_2O_3$ is multiplied by f, the result in terms of mM of chloride per liter, gm. of NaCl per liter, or mg. of NaCl per 100 cc. is found.

Example—A sample of serum was diluted to 20 times the original volume (0.5 to 10 cc.) before addition of silver iodate. Of the centrifuged supernatant liquid, 3 cc. were then analyzed, the titration requiring 2.97 cc. of the "0.03 N" $Na_2S_2O_3$, the standardized normality of which was found to be 0.03039 N. (A 2 cc. sample of exactly 0.03 N $KH(IO_3)_2$ required 1.974 cc. of the $Na_2S_2O_3$ at the end-point.) From Equation 3 the chloride content of the original sample was calculated to be

$$\frac{2.97 \times 0.03039 \times 20 \times 1000}{6 \times 0.985 \times 3} = 101.8 \text{ mM per liter}$$

Calculated according to Equation 4 and Table I, the result was found to be 2.97 × 0.03039 × 1128 = 101.8 mM of chloride per liter, or 2.97 × 0.03039 × 6599 = 595.5 mg. of NaCl per 100 cc.

Correction for Solubility of $AgIO_3$ in the Routine Method for Urine—In the *routine* analysis of urine, when the calculation according to Table I gives a result less than 60 mM of chloride per liter (or 3.51 gm. of NaCl per liter), a solubility correction for

$AgIO_3$ must be applied, exactly as for the gasometric method, according to Fig. 2 of Paper I (Sendroy (1937, *a*) p. 363).

However, in the routine analysis of such low chloride urines, *when the titration is less than 1.8 cc.*, instead of calculating according to Table I, and subtracting the correction for $AgIO_3$ solubility, we may calculate the corrected chloride concentration graphically, directly from the volume of $Na_2S_2O_3$ used for the titration. Thus, if Equation 4 be recast for this purpose,

TABLE II

Table for Direct Graphical Calculation of Titrimetric Results for the Routine Analysis of Low Chloride Urines

For supernatant samples of 3 cc. titrated with approximately 0.03 N $Na_2S_2O_3$, the titration volume, v, of the approximately 0.03 N $Na_2S_2O_3$ actually used for the titration, must first be corrected to the corresponding volume, v', of *exactly* 0.03 N $Na_2S_2O_3$, by multiplying by the standardization factor, $n/0.03$.

v' = volume of exactly 0.03 N $Na_2S_2O_3$ used	Chloride concentration of original sample, corrected for solubility of $AgIO_3$ in the supernatant	
	mM Cl per liter	Gm. NaCl per liter
cc.		
1.789	59.76	3.497
1.491	49.62	2.903
1.193	39.44	2.307
0.895	29.16	1.706
0.596	18.62	1.089
0.537	16.42	0.961
0.477	14.20	0.831
0.418	11.92	0.697
0.358	9.62	0.563
0.298	7.14	0.418
0.239	4.39	0.257
0.209	2.86	0.167

$$[\mathrm{Cl}]' = (v \times n \times f) - S \tag{5}$$

where $[\mathrm{Cl}]' = [\mathrm{Cl}] - S$ = the concentration of chloride in mM per liter, in the original sample, corrected for the solubility of $AgIO_3$ in the supernatant liquid, and S = the variable solubility correction from Fig. 2 of the preceding paper (Sendroy, 1937, *a*).

Then we may write for the routine urine analysis of 3 cc. samples of supernatant liquid, using the average K value of 0.994,

$$[\mathrm{Cl}]' = (v' \times 33.53) - S \tag{6}$$

where $v' = v \times (n/0.03)$ = volume v of approximately 0.03 N $Na_2S_2O_3$ used for titration, corrected to a corresponding volume of *exactly* 0.03 N $Na_2S_2O_3$.

Table II, calculated from Equation 6, may be used to draw curves with $[\mathrm{Cl}]'$ and v' as coordinate axes for the direct reading of corrected chloride concentrations from titration volumes of exactly 0.03 N $Na_2S_2O_3$. It is desirable that the graphs be drawn on a fairly large scale, so that there may be no great loss of accuracy in the readings of low chloride concentrations.

Examples—Two samples of urine, Samples A and B, were analyzed by the *routine* method. The supernatant samples of 3 cc. required 2.50 cc. and 0.481 cc., respectively, of 0.03 N $Na_2S_2O_3$, the standardization factor of which was 0.992. The analysis of Sample A was calculated according to Table I to be $2.50 \times 0.02976 \times 65.1 = 4.84$ gm. of NaCl per liter. The analysis of Sample B was calculated according to a chart based on Table II, whereby 0.481 cc. $\times 0.992 = 0.477$ cc. was found to correspond to a value of 0.831 gm. of NaCl per liter. Calculated according to Table I, Sample B showed $0.481 \times 0.02976 \times 65.1 = 0.932$ gm. of NaCl per liter. With the correction for $AgIO_3$ solubility from Curve B, Fig. 2, of Paper I, the final result was $0.932 - 0.104 = 0.828$ gm. of NaCl per liter.

The theoretical aspects of the reaction of Equation 1, whereby chloride is precipitated by $AgIO_3$, have already been discussed in Paper I (Sendroy, 1937, *a*). The experimental results obtained by titration have been included there and require no further discussion here.

SUMMARY

The present paper contains an outline of a titrimetric procedure for the measurement of the iodate in the chloride method outlined in the preceding paper. The IO_3^- is determined by its reaction with acidified KI, with starch as an end-point indicator: $KIO_3 + 5KI + 6H_3PO_4 = 3I_2 + 3H_2O + 6KH_2PO_4$. Thus, as in the gasometric method, the hexavalent oxidative power of IO_3^- results in 1 equivalent of Cl^- being represented by 6 equivalents of free iodine.

The accuracy and rapidity of this procedure are about the same as

of the gasometric procedure. Here, likewise, no removal of proteins, either by precipitation or digestion, from urine, or plasma or serum, is required, although protein-free filtrates of serum and of whole blood may be used for analysis.

BIBLIOGRAPHY

Conway, E. J., *Biochem. J.*, **29**, 2221 (1935).
Folin, O., and Wu, H., *J. Biol. Chem.*, **38**, 81 (1919).
McLean, F. C., and Van Slyke, D. D., *J. Biol. Chem.*, **21**, 361 (1915).
Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore (1932).
Sendroy, J., Jr., *J. Biol. Chem.*, **120**, 335 (1937, *a*); **120**, 441 (1937, *b*).
Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).

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MICRODETERMINATION OF CHLORIDE IN BIOLOGICAL FLUIDS, WITH SOLID SILVER IODATE

III. Colorimetric Analysis

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In this paper colorimetric measurement of the iodate from $AgIO_3$ in the author's method of chloride determination (Sendroy, 1937, *a*, *b*) is described. The reaction involved is the same as that of the titrimetric measurement; namely,

$$(1) \qquad KIO_3 + 5KI + 6H_3PO_4 = 3I_2 + 3H_2O + 6KH_2PO_4$$

Instead of a thiosulfate titration of the liberated iodine, however, the color of the latter is compared in a colorimeter with that of a known, standard solution of iodate, treated exactly as is the supernatant solution of the unknown from the chloride precipitation.

Colorimetric measurement of the iodate is applicable to the supernatant fluid from the $AgIO_3$ precipitation of chloride from *salt solutions* and *protein-free filtrates* (Folin and Wu, 1919; Somogyi, 1930) of plasma and of whole blood. It is not generally suitable for urine analysis, since proteins, when present, interfere.

The percentage accuracy of the colorimetric method is not equal to that of the gasometric or titrimetric methods, but the colorimetric technique can be used for the measurement of exceedingly small amounts of chloride, when the physical limitations of the other methods render them inadequate for this purpose; and the average error of the colorimetric procedure, ±1.5 per cent, permits its use for many purposes. Samples representing 0.0006 mg. of NaCl may be used for the iodate colorimetric reading, whereas at least 80 times as much is the required minimum for gasometric or titrimetric measurement.

Colorimetric methods of chloride measurement have been described by Isaacs (1922) and by Conway (1935). In Isaacs' procedure the addition of solid silver chromate causes the precipitation of the chloride and the passing into solution of chromate ion which is measured colorimetrically against suitable standards. For serum analysis Folin-Wu (1919) filtrates are used. There have been several modifications of this method, the most refined form of which is that developed by Westfall, Findley, and Richards (1934). By treating the chromate filtrate with sym-diphenylcarbazide (Cazeneuve's reagent), they obtained an increase in color intensity of 100 times. The serum analysis was confined to the use of zinc hydroxide filtrates, since the tungstic acid in Folin-Wu filtrates reacts with the Cazeneuve's reagent. Westfall, Findley, and Richards were able to analyze 0.3 c.mm. samples of serum, containing 0.01 mg. of NaCl, with an estimated average error of less than 3 per cent.

Conway's procedure involves the wet combustion of chloride in an especial apparatus, with absorption of the volatile chlorine in KI solution. In microanalyses the iodine set free is measured colorimetrically. This may be done by the use of spectral color filters for the yellow iodine color or by direct readings of the blue color developed upon the addition of starch. Conway used the latter technique for analyses at the lower limit of his method, whereby samples containing 0.01 mg. of NaCl were analyzed for chloride with an estimated average error of 6 to 7 per cent. However, he apparently did not apply the colorimetric procedure to blood and serum analysis.

In the procedure to be described the yellow iodine color may be read directly, with or without filters. However, the author prefers the color reading of the blue developed upon addition of starch. A readable yellow color is obtained for the iodine from an iodate solution diluted to 0.5 mM. By the reaction of Equation 1 the iodine obtained per mole of chloride is 6 times that obtained in Conway's method. With a further 50-fold dilution, and addition of starch, a very intense blue color is obtained which can be read even more easily than the yellow. Since the solutions used for the blue color reading represent a dilution of 1:10,000 of a 0.1 M chloride solution, and since microcolorimetric readings may be made with 1 cc. samples of solution, one is limited only by the accuracy with which small volumes of solutions and reagents may be measured and handled (Westfall, Findley, and Richards, 1934). However, for practical purposes 0.1 cc. of serum or 0.2 cc. of 1:10 serum filtrate as starting material may be considered the lower limit of sample volumes to which the colorimetric method may be applied in serum chloride analysis. Serum filtrates of either tungstic acid or of zinc hydroxide may be used. For whole blood the especial (tungstic acid + H_3PO_4) filtrate (Sendroy, 1937, *a*, *b*) is used. The colorimetric results for serum and whole blood agree with the gasometric and titrimetric values within extreme limits of ±3 per cent, with an average discrepancy of less than ±1.5 per cent.

DESCRIPTIVE

Reagents—

The preparation of the following reagents is described in the corresponding section of Paper I (Sendroy, 1937, *a*):

Approximately 0.85 M, 0.34 M, 0.17 M, 0.085 M, 0.017 M Phosphoric Acid Solution.

M/15 Potassium Acid Phosphate Solution.

Caprylic Alcohol.

Silver Iodate, Powder, C.P.

Tungstic Acid Reagent (Folin-Wu, 1919).

Zinc Hydroxide Reagent (Somogyi, 1930).

The preparation of the following reagents is described in the corresponding section of Paper II (Sendroy, 1937, *b*):

5 Per Cent Potassium Iodide Solution.

1 per cent potassium iodide solution is made from the 5 per cent solution by dilution with water.

2 Per Cent Starch Solution—While this reagent, made as described, keeps indefinitely, it is best to prepare it anew every 3 weeks when used for colorimetric work, since age seems to affect the quality of the color.

0.05 M Standard Potassium Biiodate Solution.

Standard 0.005 M, 0.001 M, and 0.0002 M solutions are made from the 0.05 M solution by dilution with water, to 10, 50, and 250 times volume, respectively.

Procedure

The general procedure, with colorimetric analysis of iodate, consists of the same steps described in the "Procedure" of Paper I on gasometric measurement (Sendroy, 1937, *a*). For the treatment of samples up to the point where the solid $AgIO_3$ and AgCl are separated from the supernatant fluid, the reader is referred to the details in the corresponding section of that paper, from steps (1) through (4), to the beginning of the description of the "Gasometric determination of total dissolved iodate."

Colorimetric Measurement

As for the gasometric and titrimetric procedures, the original sample is diluted so that the total dissolved iodate in the supernatant liquid after addition of $AgIO_3$ is between 3 and 12 mM. The volume of the supernatant fluid taken for analysis then depends on that concentration and on the amount of supernatant fluid available. Samples of from 0.02 to 0.5 cc. volume of the latter are pipetted into a suitable volumetric flask of volume such that the concentration of the iodate in the final diluted supernatant fluid approximates either 0.5 or 0.01 mM for the yellow or blue color reading, respectively. There are then added H_3PO_4 and KI solution, and the yellow iodine color is allowed to develop.

For the *yellow color reading*, dilution is then made with water to the volume mark. For the *blue color reading*, water is first added to the flask to about 0.9 volume. The capacity of the flask should be 50 times that of the flask used when the yellow color is read. Starch solution is added *slowly, with mixing*, and then water to the volume mark. The color developed is compared, in each case, with that of a simultaneously prepared standard. When blue color readings are made over a period of several hours, the inconvenience of making a new standard for each determination may in some cases be avoided by the use of one standard solution (not more than 24 hours old) with a correction for color change on standing.

The *standard* is prepared from another portion of equal volume of the same sample of the unknown which has been diluted with H_3PO_4 (in the case of salt solutions), or with protein precipitant (in the case of serum and blood filtrates), but to which *no* $AgIO_3$ has been added. Standard iodate solution is added, then the other reagents, exactly as for the analysis of the unknown.

The inclusion of the unknown solution or filtrate in the standard is necessary for accurate work, because the color developed (especially the blue with starch) is affected by the composition of the solution. With these standards, the compositions of standard and unknown color solutions are made more nearly alike. The former will, however, contain a portion of chloride salt lacking from the latter.

It is not necessary to prepare several standards of different strengths. One, of the constant iodate composition corresponding to that of a 0.1 M solution diluted 200 to 10,000 times, for the yellow or blue color, respectively, is sufficient. An approximate color match is obtained by diluting the unknown sample of supernatant fluid so that the final iodate concentration approximates that of the standard within ±20 per cent. For samples of uncertain chloride concentration, the correct dilution must first be approximated by preliminary trial and error dilutions (by varying the size of the supernatant fluid sample used), to match the color of a simple iodate standard, containing *none* of the diluted or deproteinized unknown. For samples of which the chloride concentration is approximately known, as for the filtrates of serum or of whole blood, dilution of the supernatant fluid is always made in the same way (to 20 or 1000 times volume for serum filtrates, and to 6⅔ or 333⅓ times volume for whole blood filtrates). As has already been mentioned, *in all cases exact matching of the color requires that the salts and acid in the unknown supernatant fluid and in the standard be qualitatively and quantitatively as nearly the same as possible.*

Depending on the size of the colorimeter cups used, from 1.0 to 5.0 cc. of the final solution (the diluted supernatant fluid) are needed for a determination, and a similar amount for the standard. The author has used a Bausch and Lomb colorimeter, with constant artificial light source. The standard solution is set at 25 mm. on one side, say on the right, and compared with itself on the other side, the left. The unknown is then substituted on the left side. Five or six readings of each solution against the (right) standard should be sufficient and within the range of 0.3 mm.

Specific directions for chloride determination in salt solutions, and in serum and whole blood filtrates, with colorimetric analysis of the iodate, are given below and outlined in Table I. For details of the steps leading up to the colorimetric analysis of the supernatant samples, the reader is referred to the corresponding sections in the gasometric paper (Sendroy, 1937, *a*).

A. Determination of Chloride in Inorganic Material—Samples

TABLE I

Outline of Procedure for Colorimetric Analysis of Chloride by Silver Iodate Method

Sample material	Approximate chloride concentration of diluted sample or filtrate	Volume of supernatant (after $AgIO_3$)	Volume of diluted sample or filtrate (no $AgIO_3$)	Volume of standard $KH(IO_3)_2$ solution	Volume of H_3PO_4 solution	Volume of KI solution	For color development			
							Yellow	Blue		
							H_2O to final volume	H_2O to approximate volume	Volume of 5 per cent starch solution	H_2O to final volume
Concentration of reagents				0.005 M	0.085 M	5 per cent				
	mM per l.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.	cc.
$AgIO_3$ dissolved in H_2O	0.2	5.0			0.4	0.2		90	1.0	100
Standard				0.5	2.0	1.0		450	5.0	500
Inorganic salt solution in 0.085 M H_3PO_4	10	0.5			1.5	1.0	10	450	5.0	500
Standard			0.5	0.5	1.5	1.0	10	450	5.0	500
Inorganic salt solution in 0.085 M H_3PO_4	5	1.0			1.0	1.0	10	450	5.0	500
Standard			1.0	0.5	1.0	1.0	10	450	5.0	500
Inorganic salt solution in 0.085 M H_3PO_4	3	1.5			0.5	1.0	10	450	5.0	500
Standard			1.5	0.5	0.5	1.0	10	450	5.0	500
Tungstic acid serum filtrate	7–12	0.5			2.0	1.0	10	450	5.0	500
Standard			0.5	0.5	2.0	1.0	10	450	5.0	500

Tungstic acid blood filtrate	2.6–3.8	1.5			0.5	1.0	10	450	5.0	500
Standard			1.5	0.5	0.5	1.0	10	450	5.0	500
Zinc hydroxide serum filtrate + H_3PO_4	7–12	0.5			1.5	1.0	10	450	5.0	500
Standard			0.5	0.5	1.5	1.0	10	450	5.0	500
Concentration of reagents				0.001 M	0.085 M	1 per cent				
Tungstic acid serum filtrate	7–12	0.2			0.8	2.0		180	2.0	200
Standard			0.2	1.0	0.8	2.0		180	2.0	200
Zinc hydroxide serum filtrate + H_3PO_4	7–12	0.2			0.6	2.0		180	2.0	200
Standard			0.2	1.0	0.6	2.0		180	2.0	200
Concentration of reagents				0.0002 M	0.017 M	1 per cent				
Tungstic acid serum filtrate	7–12	0.02			0.4	0.2		18	0.2	20
Standard			0.02	0.5	0.4	0.2		18	0.2	20
Zinc hydroxide serum filtrate + H_3PO_4	7–12	0.02			0.3	0.2		18	0.2	20
Standard			0.02	0.5	0.3	0.2		18	0.2	20

are diluted as described in the corresponding Section A of Paper I on the gasometric analysis. When approximately 0.1 M neutral salt solutions are analyzed, they are diluted to 10 times with 0.085 M H_3PO_4, then analyzed exactly as described for serum filtrates below. No caprylic alcohol is necessary. When supernatant samples are 5.0 or 3.0 mM in iodate, 2 or 3 times as much sample is taken as of serum filtrate supernatant liquid (see Table I).

The solubility of $AgIO_3$ (Sendroy (1937, *a*) p. 368) can easily be determined by following the outline in Table I. Samples of 5 cc. of the saturated solution are diluted to 100 cc. and compared with the 0.01 mM iodate standard, prepared as indicated. If the solubility is to be determined in a solvent other than water, the same concentration of the same solvent must be present in the standard solution.

B. Determination of Chloride in Protein-Free Filtrates of Plasma, Serum, or of Whole Blood—These filtrates are prepared for analysis as described in the corresponding Section D of Paper I (Sendroy, 1937, *a*) on gasometric analysis.

(*a*) *Tungstic acid filtrates of plasma or serum.* When enough filtrate is available,[1] samples of 0.5 cc. of the supernatant liquid are taken in a pipette, with a cotton plug if necessary, and transferred to either a 10 cc. or a 500 cc. volumetric flask. There are then added 2.0 cc. of 0.085 M H_3PO_4 solution, followed by 1.0 cc. of 5 per cent KI solution. With the 10 cc. flask, water is added to volume, and the *yellow color* is read. With the 500 cc. flask, the solution volume is increased to 90 per cent of the capacity by addition of water. Then 5.0 cc. of 2 per cent starch solution are added slowly with continuous shaking. After dilution to the mark with water, the *blue color* is read. For the *standard*, in place of the supernatant fluid, 0.5 cc. of the serum filtrate (not treated with $AgIO_3$) and 0.5 cc. of 0.005 M $KH(IO_3)_2$ are treated as above.

Analyses of 0.2 cc. and 0.02 cc. samples of supernatant liquid are outlined, together with the above, in Table I.

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[1] When tungstic acid filtrates of serum are prepared solely for use in the colorimetric chloride determination, dilution of small samples with tungstic acid reagent mixture may be made as follows: 0.5 cc. to 5.0 cc., 0.2 cc. + 2.0 cc., and 0.1 cc. + 1.0 cc. Ordinarily, the use of smaller volumes than these will not prove practicable. From the 0.5 cc. and 0.2 cc. samples, 0.5 cc. of supernatant liquid will be available for analysis, and from the 0.1 cc. samples, 0.2 cc. samples of supernatant liquid may be used.

(*b*) *Tungstic acid filtrates of whole blood* are made, and prepared for analysis as described for the gasometric method. The usual Folin-Wu filtrate may not be used. Of the supernatant fluid, 1.5 cc. are taken as sample, 0.5 cc. of 0.085 M H_3PO_4 is added, and the rest of the analysis is carried out as described for serum above. Full details are given in Table I.

(*c*) *Zinc hydroxide filtrates of plasma or serum* are made, and prepared for analysis as described for the gasometric method. Of the supernatant liquid, 0.5 cc. samples are used, with 1.5 cc. of 0.085 M H_3PO_4. The rest of the analysis is carried out as for tungstic acid filtrates, as described above. Full details for the analysis of 0.5 cc., 0.2 cc., and 0.02 cc. samples of supernatant liquid are given in Table I.

Calculations

The general equation for the calculation of the colorimetric results is

$$(2) \qquad [\mathrm{Cl}]_t = \frac{\text{reading of standard}}{\text{reading of unknown}} \times \frac{m \times d \times D}{K}$$

where

$[\mathrm{Cl}]_t$ = mM chloride per liter of original sample

m = mM concentration of iodate in the final, dilute standard solution used for the reading

d = dilution factor for the number of times the original sample was diluted with 0.085 M H_3PO_4 or protein precipitant, before addition of $AgIO_3$

D = dilution factor for the number of times the supernatant sample was diluted for the development of color

$K = \frac{[\mathrm{IO_3}]}{[\mathrm{Cl}]}$ = empirical reaction yield factor expressing the ratio of the concentration of iodate found by analysis, to the concentration of chloride originally present, per liter of sample (see "Experimental" section of Paper I (Sendroy, 1937, *a*).

For convenience in calculation, Equation 2 can take the form:

$$(3) \qquad [\mathrm{Cl}] = \frac{\text{reading of standard}}{\text{reading of unknown}} \times F$$

where

$$F = \frac{m \times d \times D}{K}$$

When results in terms of gm. of NaCl per liter, or mg. of NaCl per 100 cc. are desired, the values of F for mM of Cl per liter are multiplied by 0.0585, or 5.85, respectively.

The values of F for the several analyses outlined above are given in Table II. When the blue color is read, after the value of [Cl] has thus been found, depending on that value, a correction is applied from Fig. 1. This correction is for deviations found from Beer's law when blue color readings are made, as outlined above, against the 0.005 M $KH(IO_3)_2$ standard.

TABLE II

Table of Factors for Calculation of Results of Chloride Analyses from Colorimetric Iodate Readings, According to Equations 2 and 3

Sample material	d = original sample dilution factor	K = ratio $\frac{[IO_3]}{[Cl]}$	D = supernatant sample dilution factor	For color		
				Yellow	Blue	
				$m = 0.5$	$m = 0.01$	Correction to be made from Fig. 1, when indicated by
				Values of factor F by which to multiply ratios $\frac{\text{reading standard}}{\text{reading unknown}}$ to obtain mM Cl per liter original sample		
Inorganic salt solutions		0.996–1.000		Use Equation 2		Curve 2
Tungstic acid serum filtrate	10	1.017	20	98.3		
			1000		98.3	Curve 3
	11*	1.017	20	108.2		
			1000		108.2	" 4
Tungstic acid blood filtrate	25	1.000	6⅔	83.3		
			333⅓		83.3	Curve 1
Zinc hydroxide serum filtrate + H_3PO_4	11	0.995	20	110.6		
			1000		110.6	
	11.11	0.995	20	111.7		
			1000		111.7	

*Prepared as described in foot-note 1.

Example—A sample of 0.5 cc. of the centrifuged supernatant fluid, from the treatment of a tungstic acid filtrate (1:10) of serum with $AgIO_3$, was treated with phosphoric acid and KI and diluted to 10 cc. volume (Table I). To 0.5 cc. of a sample of the same filtrate, *not* treated with $AgIO_3$, was added 0.5 cc. of a standard solution of 0.005 M $KH(IO_3)_2$. After addition of 2.0 cc. of 0.085 M H_3PO_4 and 1.0 cc. of 5 per cent KI, this also was diluted to 10 cc. volume. Of this standard yellow color solution, 5 cc. was poured into each cup of the colorimeter. The reading on the right was set at 25.0 mm. The average of the readings on the left was 24.8 mm. The standard solution on the left was then replaced by the unknown solution, the average of the readings for which was 23.4 mm. From Equation 2 the chloride concentration of the original serum sample was calculated as

$$\frac{24.8}{23.4} \times \frac{0.5 \times 10 \times 20}{1.017} = 104.2 \text{ mM Cl per liter}$$

or more simply, from Table II, as

$$\frac{24.8}{23.4} \times 98.3 = 104.2 \text{ mM Cl per liter}$$

The same procedure was repeated, but with dilution in 500 cc. flasks, with addition of 5.0 cc. of 2 per cent starch solution after water had been added to about 450 cc. When the blue color solutions were matched, the readings were found to average 24.9 and 23.8 for standard and unknown, respectively. The original sample chloride concentration was then calculated as

$$\frac{24.9}{23.8} \times \frac{0.01 \times 10 \times 1000}{1.017} = 102.8 \text{ mM Cl per liter}$$

or

$$\frac{24.9}{23.8} \times 98.3 = 102.8 \text{ mM Cl per liter}$$

From Fig. 1 (Curve 3) a correction value of 0.7 mM per liter was added to give a final corrected value of 103.5 mM of Cl per liter.

EXPERIMENTAL

The experimental work having to do with the reaction of $AgIO_3$ with halides under many different conditions has been reported in Paper I (Sendroy, 1937, *a*). Studies of the factors affecting the colorimetric determination of the iodine obtained by the reaction of Equation 1 are given in the following.

Colorimetric Chloride Determinations, with Reading of the Yellow Iodine Color—In Table III are given the results of preliminary work on colorimetric chloride determinations.[3] Values obtained by reading the yellow colors of the iodine solutions were compared against gasometric or titrimetric analyses, the accuracy of which

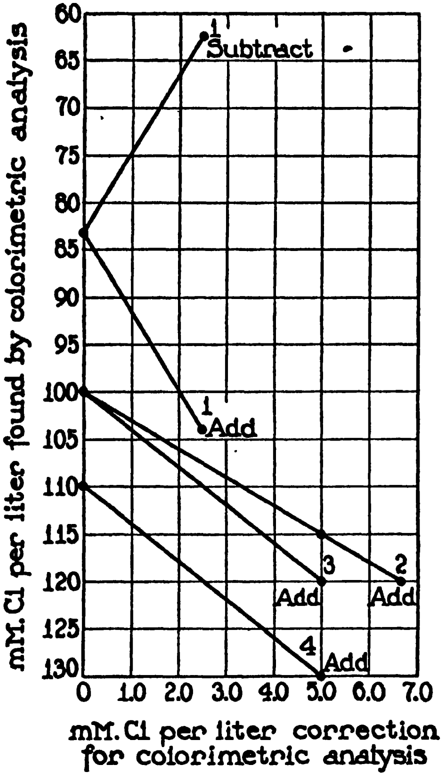

FIG. 1. Chart showing corrections to be applied to colorimetric chloride values determined by readings of the blue starch-iodine color. Curve 1, for tungstic acid + H_3PO_4 blood filtrates; Curve 2, for inorganic chloride or iodate solutions; Curve 3, for tungstic acid serum filtrates (serum diluted 1:10); Curve 4, for tungstic acid serum filtrates (serum diluted 1:11). See foot-note 3.

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[3] Table III includes one urine analysis. While the colorimetric method is satisfactory for normal urines, which are diluted so much that the yellow of the sample can be compensated for, and does not interfere, that is not the case for low chloride samples. Furthermore, if protein is present, the colorimetric method is inapplicable, without deproteinization.

has already been demonstrated (Sendroy, 1937, *a*). The results for various materials analyzed were as good as might be expected of most colorimetric procedures. To this extent they would be suitable for purposes for which no greater accuracy was required. However, yellow is not an easy color to read, and while the interposition of a blue glass filter partially overcomes this objection, it was thought desirable to develop a more sensitive color for direct reading.

TABLE III

Comparison of Colorimetric with Titrimetric and Gasometric Results by the Silver Iodate Chloride Method. Yellow Iodine Color Read

Material analyzed	d = original sample dilution factor	D = supernatant dilution factor for color reading	Chloride found		
			Gasometrically	Titrimetrically	Colorimetrically
			mM per l.	*mM per l.*	*mM per l.*
0.1 M NaCl in 0.085 M H_3PO_4	10	20			97.0
	10	20			99.4
	20	10			99.6
	25	8			100.5
	40	5			100.0
Normal urine in 0.17 M H_3PO_4	20	25		183.1	179.5
Tungstic acid serum filtrate	10	20	103.5	103.6	105.5
	10	20	102.2		104.6
	10	20	94.8	94.5	93.9
	10	20	102.5	101.7	98.2
	10	20	100.7		100.4
	10	20		105.7	110.8
	10	20	102.3		106.9
	10	20		104.3	105.7
Zinc hydroxide serum filtrate	11	20		101.2	100.7
Tungstic acid blood filtrate	20	8⅓	90.5	90.5	91.5
	25	6⅔	84.8		83.2
	11	15	82.2	82.0	82.5

Colorimetric Chloride Determinations, with Reading of the Blue Starch-Iodine Color—Although many investigators have attempted to use the blue color of the starch-iodine complex as a measure of iodine concentration in dilute solutions, their efforts have apparently met with little success. However, Turner (1930), Woodard (1934), and Conway (1935) have reported the use of starch for colorimetric iodine determinations under definite, limited conditions.

It appears from the literature and from our own experiments that so many factors affect the nature of the color produced and its sensitivity, that only under strictly controlled conditions is the starch-iodine reaction applicable to colorimetric iodate analyses. Such factors may be the concentrations of starch, of iodine, of KI, and of other salts, the degree of acidity, the presence of organic matter, and the quality of the particular starch suspension used. However, when the conditions of analysis are definitely set, and the standard and unknown prepared as alike as possible, little difficulty should be encountered. Although the authors cited above have used the starch-iodine color only in inorganic, comparatively simple solutions, the author has been able to utilize it in the presence of protein-free filtrates of serum and of whole blood, containing from 0.6 to 0.9 mg. of iodine per 100 cc. of solution.

Stability of Blue Starch-Iodine Color—In the initial experiments with iodate solution it was observed that newly prepared standards of 0.01 mM iodate (about 0.76 mg. of iodine per 100 cc.), prepared as described above, were weaker than ones which had been prepared several hours previously. This is in agreement with the observations of Turner (1930) who found that there was a slight increase in color after 16 hours. This he ascribed either to change in dispersion of the starch or to a slight oxidation of KI.

The results of our experiments in this connection are shown in Fig. 2. In five different experiments a color standard was prepared from 0.005 M $KH(IO_3)_2$ solution, as described above. In each experiment, at varying time intervals, new standards of the same strength were prepared, and their color compared with that of the first standard. The results of Fig. 2 show that, on the average, there is a general increase in color of a standard on standing for 4 hours, at the rate of about 1 per cent per hour. After 4 hours, standards have been found to be stable for about 30 hours, at least. After that time, a slight flocculation of particles has been observed.

If color comparisons against fresh standards are made within

10 minutes, the color change is imperceptible. On the other hand, if both standard and unknown are made simultaneously, and read after several hours, the color change in both will be very nearly proportional, and should not affect the results. If a large series of measurements, covering a period of several hours, is made on solutions differing in composition *only* by the amount of iodine present, one standard solution may be used. A plus correction is then made in the calculation of the unknown for increase in color of the standard, corresponding to the time elapsed between its preparation and its use against any given unknown. Thus, a simple iodate solution which matched the color of a standard prepared 4 hours previously would be calculated as being not 0.01 mM, but 0.0104 mM.

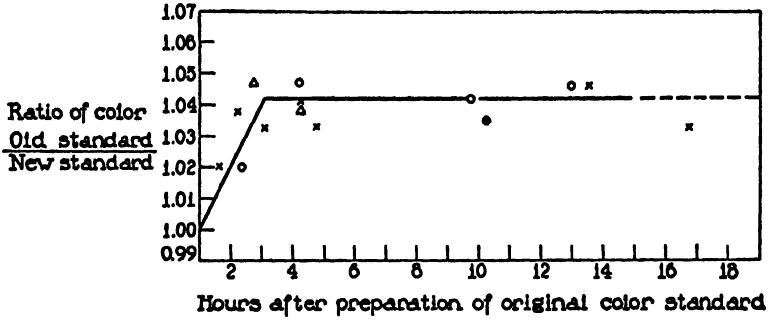

FIG. 2. The change in intensity, with time, of the blue starch-iodine color developed from standard solutions of 0.01 M iodate diluted to 1000 times volume. In each of five experiments solutions were made and the color developed anew at various intervals, to be compared with the color of the first (old) standard.

Proportionality of Color to Iodine from Inorganic Iodate Solutions and Serum and Blood Filtrates—According to Beer's law, intensity of color is directly proportional to concentration. An index of the validity of this law for any color system is the correspondence of the value for the ratio, *reading of standard:reading of unknown*, to that of the ratio, *concentration of unknown:concentration of standard*, at ratio values more or less than 1. When the one standard solution concentration of 0.01 mM iodate was used, the validity of Beer's law under the conditions of analysis described above was tested for inorganic iodate solutions and serum and blood filtrates. The deviations found were then applied as corrections for the analysis of different kinds of material.[3] The corrections are given in Fig. 1, not as deviations above and below the value of 1 for the ratio of the color readings, but in each type of analysis, for the concentrations above and below that at which the color of the material analyzed matches that of the standard. Thus from the application of Equation 2, tungstic acid blood filtrates (barring some consistent error for all determinations) match the color of the standard when the blood is 83.3 mM in chloride.

Iodate solutions of known concentrations were prepared so that they were weaker or stronger than the standard to within 20 per cent. The average results of six experiments showed that Beer's law held for solutions weaker than the standard, but not stronger. At higher concentrations the development of color was not strong enough, as is indicated by the correction Curve 2 of Fig. 1. Thus, an iodate solution of 0.125 M, diluted 10,000 times, gave a value of the ratio, *reading of standard:reading of unknown*, of 1.2 instead of 1.25.

For *serum and blood filtrates*, the criterion of deviation from Beer's law was the gasometric or titrimetric analysis of the same sample used for the colorimetric determination. The colorimetric standard used for each sample was of the same strength in iodate, 0.01 mM, and contained the appropriate filtrate as described in the procedure and outlined in Table I. *Tungstic acid filtrates of serum* (1:10) showed a minus deviation from gasometric or titrimetric results, which increased as the chloride concentration of the sample was increased, above 100 mM (Fig. 1, Curve 3). For the small samples, diluted 11-fold, the point of departure would be 110 mM, corresponding to the concentration of the original sample. The 11,000-fold dilution of such a sample would match the color of the standard 0.01 mM iodate (Fig. 1, Curve 4). *Tungstic acid* (+H_3PO_4) *filtrate of whole blood* showed a linear deviation, minus

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[3] Wright (1926–27) has called attention to the possible danger of taking too literally the colorimetric correction curves found in one laboratory, or by one individual. In the writer's opinion, if the directions given above are followed strictly in detail, no serious error should result in the use of the corrections given in Fig. 1. However, the analyst should satisfy himself on this point by making several comparative analyses such as those indicated in the following sections of this paper.

TABLE IV

Comparison of Colorimetric with Titrimetric or Gasometric Tungstic Acid Serum Filtrate Results by the Silver Iodate Chloride Method. Blue Starch-Iodine Color Read

Sample No.	Sample*	Chloride found by analysis				
		Titrimetric or gasometric	Colorimetric	Correction for colorimetric results from Fig. 1, Curve 3	Colorimetric corrected	Deviation of colorimetric from titrimetric or gasometric
		mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*
1	Pig plasma	108.2	106.9	+1.7	108.6	+0.4
2	" "	110.7	106.5	+1.6	108.1	−2.6
3	" "	115.2	112.5	+3.1	115.6	+0.4
4	Ox serum†	104.3	102.5	+0.6	103.1	−1.2
5	" "	105.5	104.6	+1.1	105.7	+0.2
6	Human serum	103.8	104.3	+1.1	105.4	+1.6
7	" "	104.3	102.5	+0.6	103.1	−1.2
8	" plasma	104.2	101.2	+0.3	101.5	−2.7
9	" "	107.6	104.3	+1.1	105.4	−2.2
10	" serum	115.3	115.1	+3.8	118.9	+3.6
11	" plasma	116.6	115.1	+3.8	118.9	+2.3
12	" "	103.0	102.0	+0.5	102.5	−0.5
13	" "	96.9	98.3		98.3	+1.4
14	" "	107.4	108.4	+2.1	110.5	+3.1
15	" serum	102.4	99.5		99.5	−2.9
16	" "	103.0	101.2	+0.3	101.5	−1.5
17	" "	80.8	82.0		82.0	+1.2
18	" "	87.0	87.1		87.1	+0.1
19	" "	90.7	90.3		90.3	−0.4
20	" "	105.6	106.1	+1.5	107.6	+2.0
21	" "	87.0	86.9		86.9	−0.1
22	" " ‡	87.0	86.5		86.5	−0.5
23	" " †	87.0	86.7		86.7	−0.3
24	" " †	105.6	103.7	+0.9	104.6	−1.0
Average..........						±1.4

Supernatant samples of 0.5 cc. were used, except where otherwise indicated.

* See foot-note 4.

† Indicates 0.02 cc. sample.

‡ Indicates 0.2 cc. sample.

above, and plus below, the critical concentration, corresponding to 83.3 mM chloride (Fig. 1, Curve 1). *Zinc hydroxide filtrates of* *serum* (+H_3PO_4) showed no consistent deviation from Beer's law in the range observed.

The colors obtained in the analysis of the serum filtrates (tungstic acid and zinc hydroxide) were a true blue, and little difficulty was experienced in matching them with that of the standards. The colors of the whole blood filtrates were slightly purplish, and sometimes did not match the standard color solutions well. Nevertheless, by making allowance for this, two analysts were able to agree well in their analyses of these samples.

TABLE V

Comparison of Colorimetric with Titrimetric Results, of Tungstic Acid (+H_3PO_4) Whole Blood Filtrate Analyses by the Silver Iodate Method. Blue Starch-Iodine Color Read

Human blood Sample No.*	Chloride found by analysis				
	Titrimetric	Colorimetric	Correction for colorimetric result from Fig. 1, Curve 1	Colorimetric corrected	Deviation of colorimetric from titrimetric
	mM per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*
1	91.4	89.5	+0.8	90.3	−1.1
2	78.8	80.5	−0.3	80.2	+1.4
3	85.9	85.6	+0.3	85.9	0.0
4	76.9	79.9	−0.4	79.5	+2.6
5	82.2	82.8	−0.1	82.7	+0.5
6	85.3	86.2	+0.4	86.6	+1.3
7	81.5	81.8	−0.2	81.6	+0.1
8	79.3	80.6	−0.3	80.3	+1.0
9	86.1	86.3	+0.4	86.7	+0.6
10	98.7	98.3	+1.8	100.1	+1.4
11	76.5	76.9	−0.8	76.1	−0.4
12	81.2	82.5	−0.1	82.4	+1.2
13	66.1	69.1	−1.7	67.4	+1.3
14	101.9	98.3	+1.8	100.1	−1.8
15	70.2	69.5	−1.6	67.9	−2.3
16	71.7	72.1	−1.3	70.8	−0.9
Average					±1.1

Supernatant samples of 1.5 cc. were used.

* See foot-note 4.

Comparison of Colorimetric with Gasometric and Titrimetric Analyses by the Iodate Method—With the average correction curves of Fig. 1, the colorimetric results were corrected and compared with values for the same samples obtained gasometrically or titrimetrically. The average deviation of the colorimetric measurements from the gasometric or titrimetric was less than 1.5 mM

TABLE VI

Comparison of Colorimetric with Titrimetric or Gasometric Zinc Hydroxide Serum Filtrate Results by the Silver Iodate Chloride Method. Blue Starch-Iodine Color Read

Human plasma Sample No.*	Chloride found by analysis		
	Titrimetric or gasometric	Colorimetric	Deviation of colorimetric from titrimetric
	mM per l.	*mM per l.*	*mM per l.*
1	118.1	121.2	+3.1
2	115.7	115.1	−0.6
3	118.1	119.3	+1.2
4	115.7	114.0	−1.7
5	86.9	85.3	−1.6
6	93.1	89.8	−3.3
7	96.6	97.2	+0.6
8	110.1	112.8	+2.7
9	116.3	115.7	−0.6
10	104.6	106.3	+1.7
11	108.1	109.2	+1.1
12	101.2	102.3	+1.1
13	103.0	103.0	0.0
14	79.8	79.0	−0.8
15	86.2	86.7	+0.5
16	89.2	89.8	+0.6
17	105.2	104.2	−1.0
18	103.0	103.4	+0.4
19†	103.0	105.1	+2.1
20	86.2	85.7	−0.5
21†	86.2	84.4	−1.8
22‡	105.2	107.0	+1.8
Average			±1.3

Supernatant samples of 0.5 cc. were used, except where otherwise indicated.

* See foot-note 4.

† Indicates 0.2 cc. sample.

‡ Indicates 0.02 cc. sample.

per liter with extremes of 3 mM per liter in the three series shown in Tables IV, V, and VI.[4] The corrected colorimetric results are all plotted in Fig. 3, against values known, or determined by some other measurement. The straight line is the theoretical path of results following Beer's law exactly.

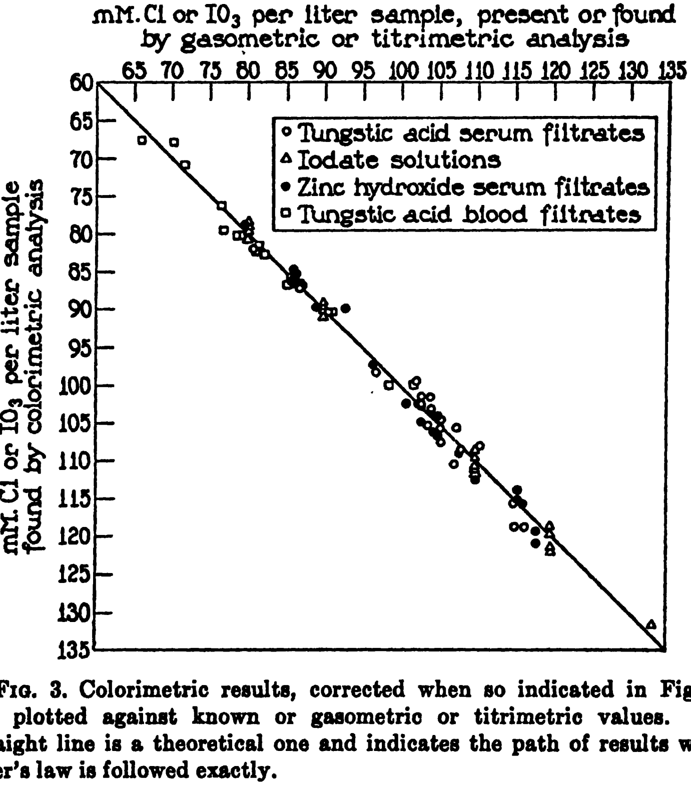

FIG. 3. Colorimetric results, corrected when so indicated in Fig. 1, are plotted against known or gasometric or titrimetric values. The straight line is a theoretical one and indicates the path of results when Beer's law is followed exactly.

From these analyses and from the evidence presented in Paper I (Sendroy, 1937, *a*) with regard to the accuracy of the gasometric and titrimetric procedures, it appears that the accuracy of the colorimetric procedure in the silver iodate method of analysis of serum and whole blood is such that the results indicate the amounts present within an average discrepancy of ±2 per cent.

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[4] The analyses of Tables IV, V, and VI are not within normal ranges. Pathological material was used, and in many cases the chloride concentration was purposely altered by addition of water or salt, to obtain extreme values.

SUMMARY

The present paper contains an outline of a colorimetric procedure for the measurement of the iodate in the chloride method outlined in Papers I and II. The IO_3^- is determined by the reaction of Paper II, with the yellow color of the free iodine, or the intense blue of the complex with starch, serving as a measure of concentration.

This procedure is not as accurate or as rapid as the gasometric or titrimetric procedure. It cannot be used in the presence of proteins, which must first be removed from urine, plasma, or whole blood. However, it is an easy procedure to follow; it requires but one standard solution and the minimum of technical ability. Furthermore, it is applicable to the analysis of extremely small samples of material.

BIBLIOGRAPHY

Conway, E. J., *Biochem. J.*, **29**, 2221 (1935).
Folin, O., and Wu, J., *J. Biol. Chem.*, **38**, 81 (1919).
Isaacs, M. L., *J. Biol. Chem.*, **53**, 17 (1922).
Sendroy, J., Jr., *J. Biol. Chem.*, **120**, 335 (1937, *a*); **120**, 405 (1937, *b*).
Somogyi, M., *J. Biol. Chem.*, **86**, 655 (1930).
Turner, R. G., *J. Am. Chem. Soc.*, **52**, 2768 (1930).
Westfall, B. B., Findley, T., and Richards, A. N., *J. Biol. Chem.*, **107**, 661 (1934).
Woodard, H. Q., *Ind. and Eng. Chem., Anal. Ed.*, **6**, 331 (1934).
Wright, S. L., Jr., *J. Biol. Chem.*, **71**, 209 (1926–27).

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NOTE ON ERRORS IN THE ANALYSIS OF CHLORIDE IN ALBUMINOUS URINE

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With the exception of the writer's silver iodate method in its gasometric (Sendroy, 1937, *a*) or titrimetric (Sendroy, 1937, *b*) form, there appears to be no method of chloride analysis in the literature which does not require the preliminary removal of proteins when present, either by deproteinization or by wet or dry ashing, if accurate results are to be obtained. Nevertheless, in the application of some chloride methods to urine analysis, steps to insure protein removal are deliberately omitted, apparently on the assumption that urine contains no protein, or so little that it need not be removed. Such an assumption is valid for normal urines, but not for the albuminous ones which make up a large part of the urines in which chloride analyses are of clinical interest. Furthermore, it is this type of urine (*e.g.*, nephritic) which is also often of low chloride content, so that the importance of errors becomes magnified.

The present critique is limited to the following methods.

Probably the most widely used method for urine chloride is *Harvey's (1910) simple and rapid application of Volhard's (1878) method.* The excess silver is titrated with sulfocyanide immediately after the precipitation, and in the presence of the chloride.

Of late, because of their obvious simplicity, *indicator adsorption methods* (Fajans and Wolf, 1924) have attracted attention in biological work. In these methods the chloride is titrated directly with standard $AgNO_3$ in the presence of an "adsorption" indicator. At the equivalence point the slightest excess of silver ion is adsorbed by the precipitated silver chloride, as is the anion of the indicator. The two adsorbed ions form the highly colored compound the appearance of which indicates the end-point.

In *Saifer and Kornblum's (1936) procedure* the urine is acidified with H_2SO_4; then diphenylamine and $K_2Cr_2O_7$ are added. Titration is carried out with $AgNO_3$ to the end-point.

In *Collier's (1936) procedure* the urine is adjusted to pH 7.0 to 7.5, then titrated with $AgNO_3$ with, dichlorofluorescein as indicator.

In the writer's *silver iodate method* (Sendroy, 1937, *a*, *b*) the urine is acidified with H_3PO_4, more or less diluted, and solid $AgIO_3$ is added. After thorough shaking and centrifugation, the dissolved iodate in the supernatant fluid is determined gasometrically or titrimetrically. Two techniques have been developed, one carried out under the optimum conditions to insure maximum accuracy, and the other in which some accuracy is sacrificed for the sake of maximum convenience and rapidity. The two procedures, "precise" and "routine," are both applicable to normal urines and to albuminous samples without removal of proteins.

As a standard of comparison Van Slyke's (1923–24) open Carius modification of the Volhard method, with nitric acid ashing, has been used.

Analysis of Albuminous Urines by the Van Slyke (1923–24), Sendroy (1937, a, b), and Volhard-Harvey (1910) Methods—Table I gives comparative analyses of albuminous urines, of low and ordinary chloride content, by several methods. As has already been shown (Sendroy, 1937, *a*), the agreement between the nitric acid digestion method, in which the proteins are digested, and the "precise" iodate method, carried out in the presence of the proteins, is good. The results by the "routine" iodate procedure are somewhat less accurate, but lie within the limits required for most clinical work.

However, serious discrepancies were found in the analyses of Samples 1 to 6, when analyzed by the simple sulfocyanate titration method. These six samples contained considerable amounts of protein and little chloride. Whether analyzed by the Volhard-Harvey method unmodified, or modified by reducing the initial amount of $AgNO_3$ used, the results obtained by two analysts, A and B, were at times not only widely divergent, but were always considerably lower than by the other two methods.

When Samples 7 to 9 were analyzed, the results agreed with the digestion and iodate methods. These samples contained little protein and a normal amount of chloride. Apparently, under these conditions, a little protein does not affect the Volhard titration. On the other hand, the inaccuracy of the method when applied to Samples 1 to 6 is not caused entirely by protein, although the large amounts present do interfere. The error in the analysis of protein-free, *low chloride* samples by this indirect method (by difference) was demonstrable even in 15 mM pure NaCl solutions for which values of 14.0, 12.3, and 13.7 mM per liter were obtained at different times, when the analyses were carried out under the conditions adopted for urine analysis.

TABLE I

Chloride Analysis of Albuminous Urines by Van Slyke (1923–24), Sendroy (1937, a, b), and Volhard-Harvey (1910) Methods

Urine Sample No.	Proteins by analysis	Chloride concentration found by analysis according to method of							
		Van Slyke	Sendroy				Volhard-Harvey*		
			Precise gasometric	Precise titrimetric	Routine gasometric	Routine titrimetric	With 5 cc. $AgNO_3$		With 10 cc. $AgNO_3$ analyzed by B
							Analysed by A	Analysed by B	
	gm. per l.	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*	*mM per l.*
1	17.0	13.2	13.0	13.1	13.2	13.5	7.9	9.1	8.2
2	19.0	12.4	12.2	12.2	11.8	12.2	6.8	8.2	5.6
3	4.7	11.5	11.5	11.4		12.4	7.7	7.5	7.4
4	31.0	11.4	11.4	11.2	11.4	11.5	3.4	7.7	4.8
5	4.9	13.1	13.1	13.1	12.1	12.7	10.6		
6	4.6	7.44	7.49	7.52		7.18	4.28	4.62	
7	0.3	155.2	155.0	154.8			151.7		153.3
8	0.5	244.2	243.6	242.7					243.4
9	1.4	210.5	209.0	207.9					207.8

* Analyses by two individuals, A and B. Analyses with 10 cc. of $AgNO_3$ were carried out as described by Peters and Van Slyke ((1932) p. 834). Analyses with 5 cc. of $AgNO_3$ were carried out in the same way, but the use of an additional 10 cc. of NH_4CNS for the titration was thereby avoided.

Analysis of Albuminous Urines by the Adsorption Methods of Saifer and Kornblum (1936) and of Collier (1936)—Table II gives the results of analyses of two samples of albuminous urine, with varying amounts of added NaCl, carried out exactly as described by the authors of the two adsorption methods used. We were unable to obtain any end-point for albuminous urine, at any concentration of chloride, by Saifer and Kornblum's method. Nor could an end-point be obtained by Collier's method in albuminous urine with the lower concentrations of chloride. End-points were observed when the chloride concentration was increased. They were not sharp, however, and the titration led to inaccurate results, unless the chloride concentration was high.

TABLE II

Chloride Analysis of Albuminous Urines by Adsorption Methods of Saifer and Kornblum (1936) and of Collier (1936)

Urine Sample No.	Protein in urine by analysis	Determination	Composition of solution titrated			Chloride concentration calculated as if all in urine		Adsorption analysis according to
			Urine	0.1 N NaCl	H_2O	Known	Found by adsorption analysis	
	gm. per l.		*cc.*	*cc.*	*cc.*	*gm. NaCl per l.*	*gm. NaCl per l.*	
6	4.6	A	1		2	0.44*	No end-point	Saifer and Kornblum
		B		1	2	5.85	5.90	
		C	1	1	1	6.29	No end-point	
		D	1	2		12.14	" "	
18	48	C	1	1	1	6.28†	" "	
6	4.6	A	1		5	0.44*	No end-point	Collier
		B		1	5	5.85	5.86	
		C	1	1	4	6.29	7.33	
		D	1	2	3	12.14	12.78	
18	48	C	1	1	4	6.28†	No end-point	

* Found by Van Slyke (1923-24) analysis.

† 0.43 gm. per liter, found by Van Slyke (1923-24) analysis of urine alone.

Effect of Aspirin Ingestion on Chloride Analyses—As noted by Jeffrey (1927), the urine of individuals who have taken aspirin shows a red tinge which interferes with the end-point observation of the Volhard titration. He suggested a suitable color control by the use of an extra urine sample. This device enables one to carry out the determination, but the end-point is still poor, and the titration inaccurate.

In one such experiment to test this point, a subject took 15 grains of aspirin in ½ hour. Urine was collected 3 hours later, colored red. By different determinations the following values for chloride were found (concentration in mM of Cl per liter): Van Slyke digestion, 229.8 (good end-point); Volhard-Harvey, 224.8 (poor end-point); Sendroy iodate, gasometric "precise" 229.8, titrimetric "precise" 229.0 (good end-point). The digestion and iodate methods were unaffected by the urine of the subject taking aspirin.

SUMMARY

Chloride titrations in albuminous urines by the Volhard and indicator adsorption methods are inaccurate and sometimes impossible to carry out, unless the protein is removed. With typical nephritic urines, low in chloride and containing protein, these methods, including the generally used Volhard-Harvey, give grossly inaccurate results. The iodate method (Sendroy, 1937, *a*, *b*) can be applied to albuminous urine without removal of the protein.

BIBLIOGRAPHY

Collier, V., Jr., *J. Biol. Chem.*, **115**, 239 (1936).
Fajans, K., and Wolf, H., *Z. anorg. u. allg. Chem.*, **137**, 221 (1924).
Harvey, S. C., *Arch. Int. Med.*, **6**, 12 (1910).
Jeffrey, W. H., *J. Lab. and Clin. Med.*, **13**, 687 (1927).
Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Methods, Baltimore (1932).
Saifer, A., and Kornblum, M., *J. Biol. Chem.*, **114**, 551 (1936).
Sendroy, J., Jr., *J. Biol. Chem.*, **120**, 335 (1937, *a*); **120**, 405 (1937, *b*).
Van Slyke, D. D., *J. Biol. Chem.*, **58**, 523 (1923–24).
Volhard, J., *Z. anal. Chem.*, **17**, 482 (1878).

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THE EFFECT OF HYPOPHYSECTOMY ON ARTERIAL BLOOD PRESSURE OF DOGS WITH EXPERIMENTAL HYPERTENSION[1]

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Experimental hypertension can be produced in animals by applying clamps to the renal arteries. This important observation of Goldblatt, Lynch, Hanzal and Summerville (1) has made it possible to study with reasonable accuracy the effects of various surgical procedures on the level of arterial pressure.

Numerous investigators, notably Cushing (2), believe that the hypophysis is concerned in the genesis of hypertension of the essential variety, and of that associated with pituitary basophilism and eclampsia. Manifestly, it is impossible in patients to ascertain the participation of the pituitary gland by its removal. But in dogs with experimental hypertension this can be done. We have therefore sought to find whether the hypophysis is concerned in the genesis of hypertension resulting from constriction of the renal artery.

METHOD. Both male and female dogs of adult ages were employed. Arterial blood pressure was measured daily by the same investigator in a warm room (80°F.). The method of Van Leersum (3) was used, which consists in ascertaining the pressure at which beats come through a loop of carotid artery compressed by a rubber pressure cuff. In a number of instances, blood pressures were also measured by auscultation over an artery of the hind leg. The artery was compressed by a 5 cm. wide rubber cuff wrapped around the leg just above the ankle joint. By this method both systolic and diastolic pressures could be determined. The two methods agree within close limits in our hands.

Blood pressure measurements were made until we felt certain that the normal variations were known. Under pento-barbital anesthesia, an adjustable silver clamp was applied to the right renal artery according to the method of Goldblatt, Lynch, Hanzal and Summerville (1). If the blood pressure rose markedly and remained steadily elevated for 30 days or more, the hypophysis was removed. But if the pressure tended to fall, a clamp was applied to the left renal artery. In some instances, it was necessary to tighten the clamp, but in most, a steady, elevated, systolic and diastolic pressure was obtained. The hypophysis was removed under visual guidance by the subtemporal approach according to the method of Sweet (4). This consists essentially of exposing the gland through a subtemporal approach, enclosing the gland within a rounded cup followed by severance of the stalk. The hypophysis was removed intact, consequently it was not necessary to kill the animal to be certain that the operation had been complete. After operation, the animals were fed large amounts of carbohydrate, or glucose was given by stomach tube if their appetite failed. Usually the animals were up and had good appetites a day or two after operation.

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[1] A preliminary report of this investigation was made in Proc. Soc. Exper. Biol. and Med. 34: 260, 1936.

The order in which the various operations were performed varied in different animals. In some, the clamps were applied and then the hypophysis removed, in others the order was reversed. In still others, after clamping and hypophysectomy, the clamps were tightened in an effort to reëstablish hypertension. In all, 17 dogs have been studied, some for two years.

The effect of thyroid, theelin and antuitrin S has also been studied on the arterial pressure of the dogs after clamping and hypophysectomy. The dosage of thyroid varied from 0.6 to 1.4 gram a day. Theelin and antuitrin S were administered intramuscularly in doses of 1 cc. a day.

RESULTS. Pressure of the clamp on the renal artery produced a marked rise in both systolic and diastolic pressure in all of the dogs, as Goldblatt, Lynch, Hanzal and Summerville (1) have shown. The elevated pressure was maintained for many weeks or months before hypophysectomy was performed. The latter operation was followed invariably by a marked reduction in arterial blood pressure. The rapidity of fall varied among the animals, but usually 10 to 20 days were required for the maximum fall to occur in both systolic and diastolic pressure. The pressure was maintained in some dogs at a level usually about 20 to 30 mm. Hg above the level normal for the individual dog (fig. 1), but in others it sank well below the normal level (fig. 2). For example, the systolic pressure fell as low as 76 mm. Hg and remained low for several days, yet the animal appeared to be in excellent health. In order to be certain that the operation alone, without removal of the hypophysis, did not affect the arterial pressure, a control operation was performed on one dog and 12 days later the gland was removed by approaching it from the opposite side. No fall in pressure resulted when the gland was not removed, but removal caused the usual reduction in pressure.

Four normal dogs were subjected to hypophysectomy to ascertain the effect on the normal blood pressure. Two of them exhibited a fall from an average level of 130 mm. Hg to a level of 110 to 120 mm. (confirming the results of Braun-Menendez, 5), while the pressure in the other two did not appear significantly altered.

Among the 17 dogs studied, marked variability was observed in response of the arterial pressure to hypophysectomy and subsequent increase of renal arterial constriction by the clamp. The arterial pressure fell to levels well below normal in 5 of the dogs and remained so, except for minor interludes, for periods up to 2 years. In these animals it has been impossible to produce more than a transient rise in pressure by further constricting the renal artery. Clinically, they were characterized by the facts that they all increased markedly in weight (one rose from 13.5 kgm. to 30.0 kgm.), they were docile, quiet and somewhat sluggish, their appetites were excel-

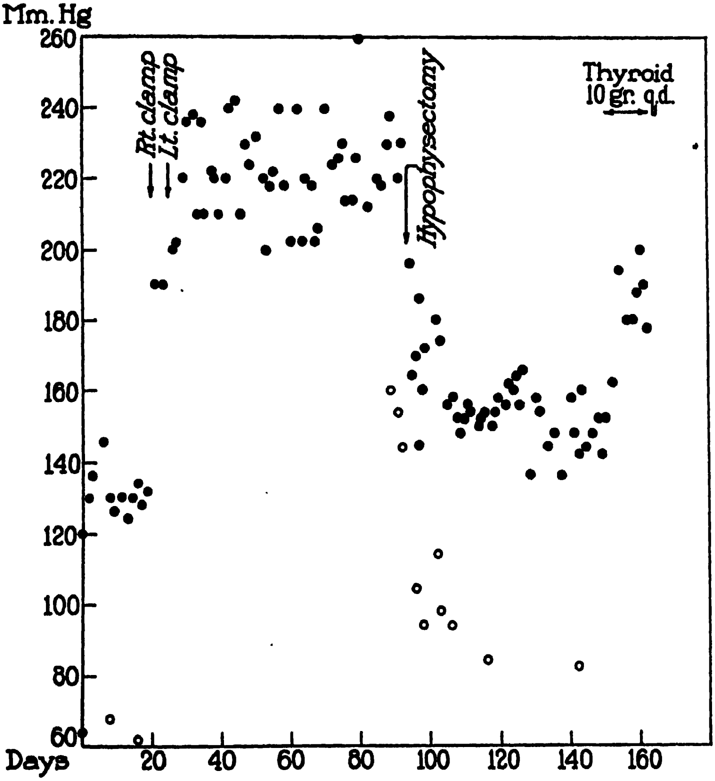

Fig. 1. Effect of hypophysectomy on systolic (solid dots) and diastolic pressure (circles) of dog with hypertension.

lent, their coats were soft, luxuriant and tended to shed and be replaced frequently, and they all had polyuria. The basal metabolic rate was

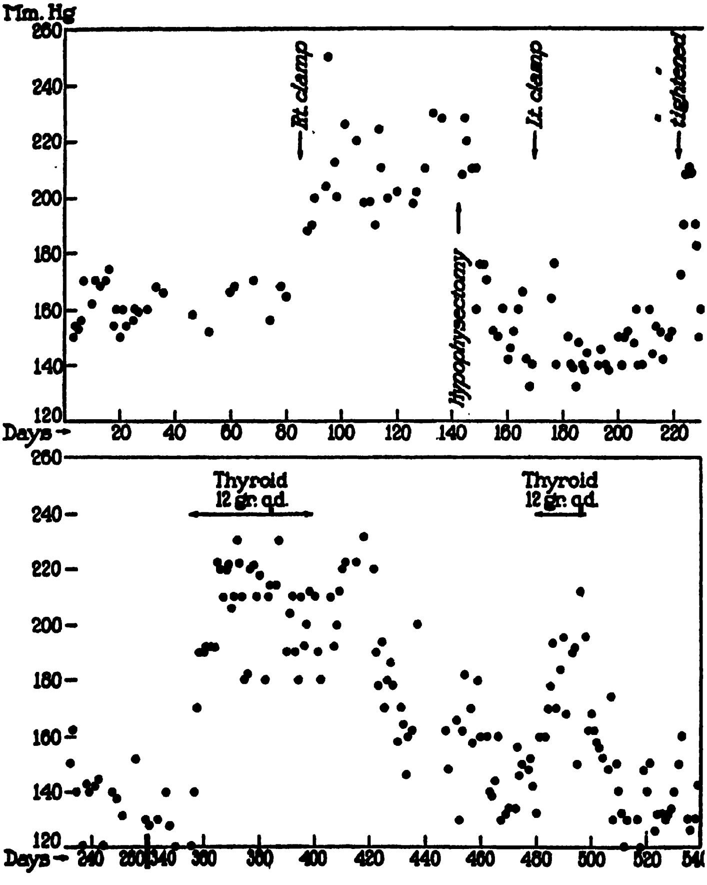

Fig. 2. Effect of hypophysectomy and thyroid feeding on arterial pressure of a dog. A portion of the chart has been deleted (between 260 and 340 days) which represented daily blood pressure measurements varying from 98 to 140 mm. Hg for a period of 80 days.

measured in one animal of this group and was found reduced (13 per cent of normal).[3]

Contrasting with this group of animals were those in which the fall in blood pressure seldom was less than the normal level for the dog and usually was 20 mm. or more above it. Increase in the constriction of the renal artery subsequent to the hypophysectomy usually resulted in a definite and sometimes sustained rise in arterial pressure. These animals were clinically easily distinguishable from the first group. They were either lean or well-nourished, but not fat. They were active, alert, and in some cases excessively nervous. The blood pressure level was highly labile.

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[3] We wish to express our appreciation to Dr. D. Holman for making these measurements.

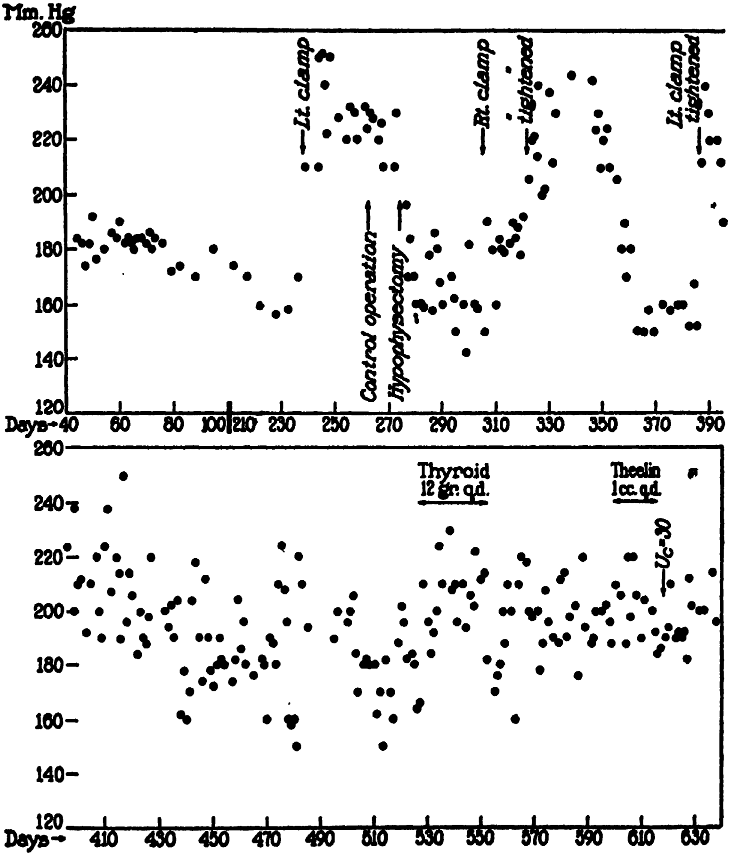

Fig. 3. Effect of control operation followed 12 days later by hypophysectomy on blood pressure of a dog with hypertension. Later both clamps were further tightened. The effect of thyroid feeding and theelin injection is also shown. The record covering a one hundred day period during which daily blood pressure measurements were made has been deleted from the chart of the preoperative control period.

No change was observed in their coats. Polyuria was present less often. The basal metabolic rate of one of the dogs in this group was as high as +50 per cent of normal. However, due to the nervousness of the animal it is probable that the measurements were not made under basal conditions.

It should be noted that the general health of both of these groups of animals appeared to be excellent. Both male and female animals were in each group. The differences lay chiefly in their behavior.

Both groups of animals responded to large doses of thyroid substance by rise in arterial pressure. Thyroid was not administered until several months after hypophysectomy, so that time was allowed for the involutional changes in other organs to occur which result from removal of the pituitary gland. But the pressure never rose to the original high level even after feeding thyroid for 3 to 5 weeks. The dogs became very nervous and overactive but the rise was seldom greater than 80 mm. Hg. As a control, two normal dogs were given the same amount of thyroid as the hypophysectomized animals. The average blood pressure rise was not greater than 20 mm. in them. Theelin and antuitrin S produced no change in the average blood pressure level in either normal or hypophysectomized dogs.

Discussion. Hypophysectomy reduces the blood pressure of adult hypertensive dogs to levels which are either normal or somewhat above normal. The reduction usually occurs during the course of 20 days or less after operation. Trephining the skull and retraction of the brain, but without removal of the gland, does not lower the arterial pressure; consequently, the hypophysis appears to be responsible for the reduction of the blood pressure level. But if the pressure is lowered as a result of the hypophysectomy, it may be again elevated, if at times only temporarily, by tightening the clamps on the renal arteries or clamping the remaining renal artery if the hypertension has been produced by application of the clamp to only one artery.

We interpret these results in the following way. Hypophysectomy does not remove an organ which is secreting a pressor substance directly responsible for the hypertension following constriction of the renal artery. Were this so, it might be expected that the blood pressure would fall much more quickly than it does, and that the fall would be to normal, neither above nor below it. All of the animals should respond in essentially the same manner, and increase in the severity of the renal artery constriction after hypophysectomy should no longer produce a rise in blood pressure. Hypophysectomy performed a month or more before the renal arteries were constricted should prevent the hypertension ordinarily resulting from this procedure. The results were contrary to these expectations.

Removal of the hypophysis is well known to affect a number of organs with which it is closely associated in normal metabolism. The chief among these are the thyroid gland, the adrenal glands, and the gonads. Atrophic changes with diminished function of these glands has been amply proven. Van Dyke (6) has recently reviewed this evidence. Administration of thyroid would thus be expected to aid in restoring the pressure to its elevated level. This was in fact observed, though the response was not usually great, even with doses of thyroid far greater than physiological. Among the remaining glands known to undergo involution after hypophysectomy, it would be anticipated that the adrenal gland would be most important in the genesis of hypertension because of the very low blood pressure which results from either disease or as a result of its extirpation. Indeed Goldblatt has very recently reported in a lecture (10) that bilateral adrenalectomy was followed by a sharp fall in blood pressure from the elevated to normal levels. One of us (I. H. P.) has confirmed this in 6 hypertensive animals.

It is, then, our opinion that most evidence favors the belief that hypophysectomy influences the arterial pressure level of hypertension in dogs indirectly through loss of its stimulating effects on other endocrine glands, chiefly the adrenal cortex and less importantly the thyroid. Whether other organs such as the liver are also affected is not known. It appears certain that the nervous system is not an essential link in the mechanism, for, as Freeman and Page (7) showed, hypertension persists after complete removal of the sympathetic nervous system, combined with denervation of the heart. Nor does section of the splanchnic nerves and removal of the lower thoracic ganglia affect the blood pressure (Goldblatt, Gross and Hanzal, 1). Furthermore, Glenn, Childs and Page (9) found that the blood pressure tends to return to elevated levels in hypertensive dogs even after complete destruction of the spinal cord below the fifth cervical segment.

Our experiments do not eliminate the possibility that injury to the diencephalic vegetative centers adjacent to the hypophysis may also have some influence on the response attributed to hypophysectomy. Indeed the fact that some of the dogs became excessively fat suggests that injury had occurred. One of us (I. H. P.) is investigating this problem in hypertensive cats.

We therefore conclude that hypophysectomy alters the reactivity of the vascular system by diminishing the activity of the adrenal cortex, thyroid and perhaps other organs in such a manner that it no longer responds as well to chemical stimuli resulting from constriction of the renal artery.

SUMMARY

1. Hypertension of the order of 240/160 mm. Hg was produced in dogs by constricting the renal arteries of dogs by means of Goldblatt's clamp.

It was maintained for several months. Hypophysectomy in these animals reduced the arterial pressure to levels slightly above normal (150/100 mm.) or below normal (90/40 mm.) within a period of 20 days. Hypophysectomy in normal dogs reduced the arterial pressure only slightly (from about 140/70 to 116/50 mm.).

2. Increasing the constriction of the renal arteries after hypophysectomy again produced a rise in blood pressure, but this tended to be less marked and transient, especially in the dogs which became sluggish and fat and exhibited reduced basal metabolism and often diabetes insipidus. The rise was better maintained in dogs which were thin and active and with normal or elevated basal metabolism.

3. After hypertension had been reduced by hypophysectomy, feeding thyroid (0.8 gram) raised the blood pressure moderately (190/120 mm. Hg), and injection of theelin (1 cc. daily) or antuitrin S (1 cc. daily) had no effect.

4. The effect of hypophysectomy on hypertensive dogs is believed to be an indirect one. It is postulated that the responsiveness of the blood vessels to chemical stimuli from the kidneys with constricted renal arteries is reduced. This may be due to lack of the secretions of the adrenal and thyroid glands. Deficiency of these secretions may in turn be due to withdrawal by hypophysectomy of the chemical stimuli normally afforded them by the hypophysis.

REFERENCES

(1) Goldblatt, H., J. Lynch, R. F. Hanzal and W. W. Summerville. J. Exper. Med. 59: 347, 1934.
(2) Cushing, H. Arch. Int. Med. 51: 487, 1933.
(3) Van Leersum, E. C. Pflüger's Arch. 142: 377, 1911.
(4) Sweet, J. E. Ann. Surg. 102: 1069, 1935.
(5) Braun-Menéndez, E. Rev. Soc. Argent. Biol. 8: 463, 1932.
(6) Van Dyke, H. B. The physiology and pharmacology of the pituitary body. Univ. Chicago Press, Chicago, 1936.
(7) Freeman, N. E. and I. H. Page. Am. Heart, J. 1937 (in press).
(8) Goldblatt, H., J. Gross and R. F. Hanzal. J. Exper. Med. 65: 233, 1937.
(9) Glenn, F., C. G. Childs and I. H. Page. Unpublished observations.
(10) Goldblatt, H. J. A. M. A. 108: 675, 1937.

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PHYSIOLOGICAL PROPERTIES OF A CENTRAL EXCITATORY AGENT IN FLUID OBTAINED BY OCCIPITAL PUNCTURE OF MAN AND ANIMALS

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Extracts of fluid obtained by occipital puncture in man have been reported to cause marked rise in arterial pressure when injected into cats which were lightly anesthetized and with their vagi severed (Page, 1935). The pressor action was not observed when the central nervous system was destroyed, or when its function was impaired. Consequently, it was concluded that the nervous system was an essential link in the pressor mechanism. Further studies on the physiological properties of these extracts have been made and are reported in this communication.

Since such extracts stimulate other nuclei in the brain than those concerned with the pressor mechanism, such for example as the vagus nuclei, it seems desirable to call the active substance (or substances) central excitatory. For convenience then, the active material in extracts of fluid from the cisterna magna and the third ventricle will be called a "central excitatory agent" (abbreviated C. E. A. in this communication). By this connotation it is meant to avoid any implication that it is physiologically active.

METHOD. The method employed to study the action of extracts was the same as that previously described (1935). In essence it consisted in adding the fluid to be tested to alcohol in the proportion of 1 to 50, filtering off precipitated protein after it has stood in an ice box, and removing the alcohol under reduced pressure. Removal of the alcohol usually caused the volume of the original fluid to be reduced to one-half, but not more and *usually less*. The extracts were water-clear and gave no precipitate with 95 per cent alcohol. This aqueous residue was injected intravenously into cats. The animals were prepared by lightly anesthetizing with ethyl urethane and four to five hours later both vagi were severed. Arterial pressure was recorded by a mercury manometer from the right carotid or femoral artery. Heparin was used as anticoagulant in the cannula and in the tubing connecting it with the manometer. The effect of the volume of the injection was estimated by injecting the same amount of warm physiological saline solution before making the test injection. Usually the volume was less than 2 cc.; consequently, it did not affect the blood pressure significantly.

Fluid was obtained from patients by occipital puncture within as short a period after death as possible.[1] This varied from 10 minutes to 24 hours. Fluid was also obtained from the third ventricle and cisterna magna after the brain had been opened. Occipital punctures were performed also on living animals and the first 1 to 2 cc. of fluid employed. Some of the dogs had normal blood pressures, and some had been made hypertensive by constricting the renal arteries (method of Goldblatt, Lynch, Hanzal and Summerville, 1934).

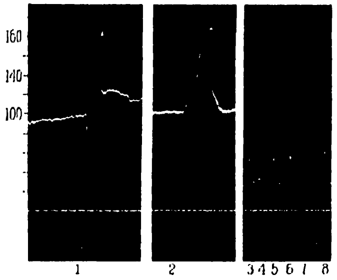

Fig. 1

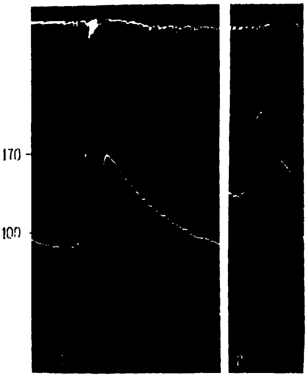

Fig. 2

Fig. 1. Pressor effect of cisternal and spinal fluid extracts from patient suffering from malignant hypertension (B. P. = 230/176). *1.* Extract of 0.5 cc. of fluid obtained by occipital puncture. *2.* Alcohol extract of 1.2 cc. spinal fluid. *3.* Pithed cat; 1 cc. of 1:100,000 adrenalin. *4.* Alcohol extract of 3 cc. of spinal fluid. *5.* Adrenalin. *6.* Adrenalin. *7.* Extract of 5 cc. spinal fluid. *8.* Adrenalin. Time marker represents base line. Each grouping of 10 vertical lines represents 1 minute.

Fig. 2. Effect of extract of occipital fluid of a normal anesthetized dog on blood pressure and nictitating membrane of an adrenalectomized cat injected with cocaine (4 mgm.). Upper line represents respiration; middle line the movements of the nictitating membrane; lower line of the arterial pressure. At *1* and *2* extract of 1.5 cc. of occipital fluid was injected.

Results. *Effect on anesthetized cats with both vagus nerves severed. Character of the pressor response.* The pressor response was initiated usually by a slight fall in blood pressure which was followed by a sharp rise about 10 seconds after completion of the injection. During the ascent, the pressure might reach a plateau followed again by a still higher rise. It then fell in from 2 to 3 minutes to the original level. Less frequently it required 4 to 16 minutes, or more, to return to normal. The plateau in the curve might not occur, nor was the preliminary short fall in pressure always seen.

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[1] I wish to express my appreciation to Dr. Milton Helpern of Bellevue Hospital for his aid in obtaining some of these fluids from cadavers and to Dr. Charles Elsberg and Dr. Byron Stookey for fluid from the lateral ventricles of patients.

Effect on nictitating membrane. C. E. A. caused contraction of the nictitating membrane in cats (fig. 2). Contraction often occurred even when arterial pressure fell instead of rising. But the extent of contraction was usually not as great as that elicited by injection of epinephrine. It was much less than the powerful contraction of the membrane produced by tyramine.

Sensitization of the nictitating membrane to epinephrine was secured in two ways, namely, denervation by removal of the superior cervical ganglion (Meltzer and Auer, 1904) and by injection of cocaine (Rosenblueth and Cannon, 1932).

The membranes were found very sensitive to epinephrine eight days after denervation, but C. E. A. failed to cause immediate contraction. When the arterial pressure fell, or toward the end of the pressor response, a small contraction of the membrane was obtained. It was believed that this was due to secreted epinephrine. Membranes sensitized by intravenous injection of cocaine responded moderately to stimulation by C. E. A. but the response was less marked than before the injection of cocaine.

Repeated injections of C. E. A. caused the normal membrane to respond more and more sluggishly until it ceased to respond altogether. Injection of epinephrine or tyramine at this time still caused a sharp contraction of the membrane.

In two experiments it was found when ergotoxin had reversed the pressor action of epinephrine on arterial pressure to depressor, and the membrane still contracted, that C. E. A. no longer caused contraction. This action is possibly due to toxic action of the drug on the central nervous system.

The facts that C. E. A. ceased to stimulate contraction when epinephrine and tyramine still caused powerful contraction, that denervation abolished the response, that cocaine decreased rather than increased the sensitivity of the membrane, and that the membrane was so easily "fatigued" by C. E. A., suggested that stimulation of it was mediated through the nervous system. This contrasted with the direct action of epinephrine and tyramine.

Certain effects on respiration, renal volume and the eyes. The response of respiration to C. E. A. varied greatly in the same and in different cats. Often there was immediate increase in rate and depth followed by a short period of standstill. The increase might not appear and about 30 seconds after the maximum rise in arterial pressure had occurred the respiration became shallower until standstill occurs, followed in another 30 seconds by resumption of the original rate and depth. Still another common type of response was marked increase in rate and depth without respiratory standstill. There appeared to be no correlation between the respiratory response and the response of arterial pressure.

Four experiments were performed in which the right kidney of the cat was placed in a Livingston oncometer connected with Brodie bellows. Rise in arterial pressure was usually accompanied by sharp reduction of renal volume, but even if a fall in pressure resulted from the extract, renal volume was reduced. Removal of the kidneys, intestine, liver, adrenals, spleen and pancreas did not, however, prevent a rise in arterial pressure by C. E. A. (2 experiments).

It was often noticed that when the response to C. E. A. was powerful the eyes of the anesthetized animal appeared to bulge from their sockets and extreme miosis occurred. Both quickly subsided.

Effect of C. E. A. on blood pressure and heart rate before and after section of the vagus nerves. When C. E. A. is injected before the vagus nerves are severed, a transient and, at times, prolonged fall in blood pressure occurs. The heart rate is sharply reduced. If the pressure is allowed to return to its original level and then the vagi are severed quickly, the arterial pressure rises rapidly, often as much as 120 mm. Hg. The rate of the heart beats increases markedly. For example, in one experiment the blood pressure fell 74 mm. Hg. and the heart rate decreased 60 beats per minute. As the rise in pressure to its original level occurred both vagus nerves were cut. The blood pressure rose 154 mm. Hg and the heart rate increased 100 beats per minute.

The same experiment has been repeated four times using histamine to reduce the blood pressure. In two of the experiments, when the pressure was reduced 92 and 96 mm. Hg respectively, no change in heart rate occurred. Section of the vagi caused increases of 37 and 90 beats per minute. In the third experiment the heart rate increased 12 beats per minute while the blood pressure fell 52 mm. Hg; the rate then rose 30 beats after section of the vagi. In the fourth experiment, the heart rate fell 40 beats with a concomitant fall of 72 mm. Hg in arterial pressure. The rise in pressure after vagus section was less marked when histamine was used to alter the blood pressure than with C. E. A.

If, after the return of pressure to normal levels following section of the vagi, more C. E. A. is injected, only a slight, if any, fall in pressure occurs, and the blood pressure rises sharply and markedly. The heart rate is usually not greatly changed even at the peak of the pressure. In most of the experiments, a rise of from 2 to 30 beats occurs.

When the vagi are intact, they restrain markedly the rise in arterial pressure which follows injection of C. E. A. In some experiments this restraint appears to be so great that only a moderate rise occurs after a

period of reduced pressure and slow heart rate. Liberation from the restraint due to the vagi is followed by a sharp rise in pressure. From these experiments it is concluded that the nuclei of the vagus nerves are strongly stimulated by C. E. A. and that these nerves act to restrain the simultaneous sympathetic stimulation. The carotid sinus mechanism does not appear to play a predominant part in the inhibition because in three experiments the blood pressure rose sharply when, prior to the injection, both vagi were cut and both carotid arteries tied off above and below the sinus. If, however, the vagi were left intact and both carotid arteries tied off, vagus inhibition occurred as when the arteries were not occluded. It is not meant to imply that the carotid sinus mechanism does not influence the response to C. E. A. but simply that response may occur even when it is inactivated.

Effect of destruction or reduction of the function of the nervous system. After it had been shown that the cat used for the experiment responded actively to C. E. A., the carotid arteries were tied off and it was quickly pithed through a trephine hole in the skull thus destroying brain and most of the cord. Time was allowed until the blood pressure had assumed a constant level and the pressure had been shown to respond to small doses of epinephrine. The same amount of C. E. A. employed before pithing was then injected. It was invariably found that no noteworthy response was obtained (fig. 1).

Reduction of nervous function by anesthesia also prevented the response. If the animals were so deeply anesthetized that they either did not respond, or responded only sluggishly to inhalation of 10 per cent CO_2-air mixture, or to stimulation of the carotid sinuses by occlusion of the common carotid arteries, the response to C. E. A. was either minimal or failed entirely. For this reason, failure to obtain a response does not always indicate that the extract is inactive. Experience has taught that the best time for obtaining active responses is from 4 to 5 hours after intraperitoneal injection of 6 cc. of a 25 per cent solution per kilogram of ethyl urethane.

The effect of anesthesia on the C. E. A. response is similar to the effect on the response of repeated injections of C. E. A.

Successive injections of C. E. A., even if ample time was allowed for the blood pressure to return to the pre-injection level, was followed rapidly by progressively weaker responses, until not only is the pressor action abolished, but sharp depression of arterial pressure occurs. Cats were occasionally found that responded to C. E. A. only by reduction of the pressure level. Quantitative assay by this method was consequently very difficult (fig. 3).

Effect of adrenalectomy and splanchnic resection on the pressor response. The occurrence of a plateau in the curve of arterial pressure suggested that after the initial rise the secondary one was due to secretion of stimulated adrenal glands. Bilateral adrenalectomy using the posterior approach was performed both one hour before the injections were made and directly after a pressor response had been elicited. Seven experiments were carried out (fig. 4). The operation usually modified the response somewhat in that the rise in pressure was not as marked. In two experiments, the same extracts which had caused rise in pressure caused a sharp fall after adrenalectomy. However, in most experiments, the pressor response was still obtained and even the plateau was observed. While adrenalectomy appeared to reduce the intensity of the pressor response, it did not abolish it, and consequently could not be held responsible for the chief action of the extracts. To this evidence must be added the fact that pithing abolishes the response completely. If it were assumed that C. E. A. had a direct action on the adrenal glands, this operation should not interfere with the response. These experiments suggest that, although the character of the blood pressure response is somewhat altered by removing the adrenal glands, they are not necessary for it.

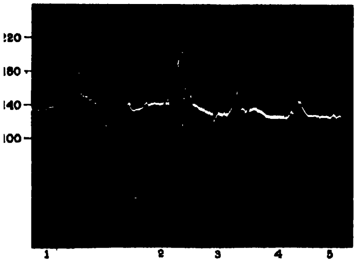

Fig. 3

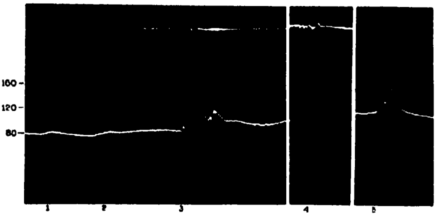

Fig. 4

Fig. 3. Effect on cats' blood pressure of repeated injections of extract of ventricular fluid from patient with chronic nephritis and hypertension (Hosp. no. 8295). *1.* Extract equivalent to 2 cc. of ventricular fluid. *2.* Same, equivalent to 3 cc. *3.* Same, equivalent to 2 cc. *4.* Same, equivalent to 2 cc. *5.* Same equivalent to 4 cc. ventricular fluid.

Fig. 4. Effect of suprasellar cyst and ventricular fluid extract from a patient suffering from a craniopharyngioma on cats' arterial pressure. *1.* Five cubic centimeter of saline. *2.* Ten cubic centimeters of fresh ventricular fluid. *3.* Extract of 0.5 cc. of fluid from the cyst. *4.* Extract of 3 cc. of ventricular fluid. *5.* Extract of 1 cc. of cyst fluid injected after bilateral adrenalectomy. The upper line represents respiration.

In one experiment the splanchnic nerves were resected just below the diaphragm, partially denervating the adrenal glands. The response to C. E. A. was as great after the operation as before. This emphasizes again that the adrenal glands are not essential for the pressor response.

Effect of ergotoxin,[2] *cocain, and atropin on pressor response.* Were the pressor action due to liberation of epinephrin, it might be expected that ergotoxin would reverse it from pressor to depressor, just as is observed when epinephrine is injected (Dale, 1906). Experiments were performed in which sufficient ergotamine tartrate was given to cause sharp depression of arterial pressure when epinephrin was injected in doses which prior to the ergotamine produced a rise in pressure. The response to C. E. A. was often reduced in intensity but reversal did not occur. Diminution in magnitude of the response may be only partially due to ergotamine because the second dose of extract always produces a less active response than the first (fig. 5, nos. 3 and 6, and fig. 5, nos. 6 and 11).

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[2] Ergotamine tartrate-Sandoz Chemical Company.

Atropin in doses sufficient to reverse the depressor action of choline to pressor also did not appear to have any characteristic effect on the response to C. E. A. (fig. 5, nos. 8 to 11 for example.)

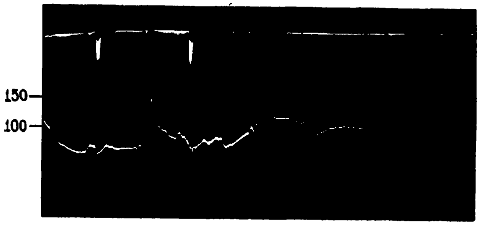

Fig. 5. Effect of ergotoxin and atropin on pressor action of extract of occipital fluid of dogs. *1.* Adrenalin 0.5 cc. of 1:100,000. *2.* Saline 2 cc.—animal struggled. *3.* Extract of fluid from normal dog 0.5 cc. *4.* Ergotamine tartrate 1 cc. of 1:2000 solution. *5.* Adrenalin. *6.* Same extract as (*3*). *7.* Adrenalin. *8.* Choline 1.5 mgm. *9.* Atropin grain 1/50. *10.* Choline 1.5 mgm. *11.* Same extract as *3*, upper line represents respiration.

Cocaine, as shown by Fröhlich and Loewi (1910) produces marked potentiation of the pressor action of epinephrin. After such potentiation had been secured and a dose of C. E. A. had been given as a control, the same C. E. A. was retested. It was found that cocaine had no effect on the response. Occasionally, it was observed that the response was reduced. This was believed to be due to the toxic action of cocaine on the nervous system, as in some of these experiments other signs of intoxication were noted such as irregular rhythm of the heart.

Comparison of epinephrin and C. E. A. on arterial pressure. A series of 4 experiments have been performed to ascertain whether the response to C. E. A. varies in some regular fashion with the response to epinephrin. These experiments leave no doubt that parallelism does not exist. A strong pressor response can be elicited by C. E. A. at times when the animal responds by fall of blood pressure to the dose of adrenalin which usually causes a sharp pressor action.

Sources of C. E. A. It may be of interest to mention briefly some of the sources of fluids from which C. E. A. was prepared. Fluid obtained by occipital puncture from patients suffering from a variety of diseases have yielded it. These fluids are by no means limited to patients suffering from hypertension. Occipital puncture in dogs both before and after hypophysectomy yield it. Consequently, the hypophysis is not responsible for the genesis of the active substance. C. E. A. may be obtained from occipital fluid from normal cats and goats.[3] Fluid obtained at operation from a suprasellar cyst also yielded it.

Fluid obtained by spinal puncture or from the lateral ventricles of the brain usually yields extracts, in our experience, which contain only very small amounts of C. E. A., comparable in amount to that in blood. Consequently, only the first portion of fluid removed from the cisterna is employed in the preparation of extracts. In cats, for instance, only the first 1 or 2 cc. of fluid contain the active material. Only under circumstances, not as yet clearly defined, does fluid from the lateral ventricles and the spinal canal yield C. E. A. in amounts which appear to be unusual.

Since C. E. A. may be obtained from a variety of animals and men during both normal and morbid states, the substance or substances apparently do not appear alone during disease.

It appears to be partially bound to some substance in the fluid contained in the cistern, because injection of fresh fluid without preliminary treatment with alcohol causes a rise in blood pressure usually less than 20 per cent of the rise when the same fluid is treated with alcohol. Its combination with other substances must be very weak to be broken by such gentle treatment.

SUMMARY

1. Fluid obtained by occipital puncture from dogs before and after hypophysectomy, cats, goats and man, yields an extract which when injected into anesthetized cats produces physiological evidence of marked central excitation. If the vagi are intact the blood pressure falls and the heart is markedly slowed followed later by a rise in pressure and increase in heart rate. If the vagi are severed, injection of C. E. A. (abbreviation for a central excitatory agent) is associated with a sharp rise in blood pressure without significant change in heart rate (fig. 1). Simultaneously exophthalmos, retraction of the nictitating membrane and other evidences of excitation occur.

2. The excitation appears to be mediated by the nervous system because *a*, injury to or removal of the central nervous system abolishes the response; *b*, the response is rapidly fatigued (fig. 3); *c*, adrenalectomy, splanchnic

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[3] An extract of 4 cc. of fluid from a goat raised a cat's blood pressure 98 mm. We wish to thank Dr. J. Beard for the occipital fluid of goats.

nerve resection and carotid sinus denervation may modify somewhat but do not inhibit completely the response; *d*, the response differs in several respects to peripherally acting substances such as epinephrin and tyramine.

3. The pressor response is probably not due chiefly to epinephrin secreted by the adrenal glands because *a*, adrenalectomy alters but does not abolish the response (fig. 4); *b*, control periods of asphyxia do not duplicate the response; *c*, cocain does not potentiate the response either of the blood vessels or the nictitating membrane; *d*, ergotoxin in doses sufficient to reverse the pressor response of epinephrin to depressor do not reverse that of C. E. A. (fig. 5); *e*, denervation of the nictitating membrane by superior cervical ganglionectomy abolishes the immediate response to C. E. A. but sensitizes it to epinephrine; *f*, repeated doses of C. E. A. rapidly diminish the response of the mechanism responsible for the rise in arterial pressure contrary to the action of epinephrin (fig. 2).

REFERENCES

Page, I. H. Science 82: 550, 1935.
J. Exper. Med. 61: 67, 1935.
Goldblatt, H., J. Lynch, R. F. Hanzal and W. W. Summerville. J. Exper. Med. 59: 347, 1934.
Meltzer, S. J. and C. M. Auer. This Journal 11: 28, 1904.
Rosenblueth, A. and W. B. Cannon. This Journal 99: 398, 1932.
Dale, H. H. J. Physiol. 34: 163, 1906.
Fröhlich, A. and O. Loewi. Arch. f. exper. Path. u. Pharmakol. 62: 159, 1910.

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THE EFFECT OF PROLONGED LOW PROTEIN DIET ON THE SERUM LIPIDS OF DOGS

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In the nephrotic type of Bright's disease two of the outstanding phenomena are the increase in plasma lipid concentration and the decrease in plasma proteins. There is also as a rule a marked loss of tissue protein, so that if edema disappears, the muscles are seen to be wasted; there is a state of general protein malnutrition. The question naturally arises whether the hyperlipemia is induced by the protein malnutrition.

Against such an origin of the hyperlipemia are the quantitative results of Page, Kirk, and Van Slyke (6), who found "no parallelism in severity between lipemia and plasma protein deficit, although both accompany the nephrotic syndrome." These findings have been confirmed by more extensive studies of Page and Farr (5). Furthermore, in subjects without renal disease, but suffering from hypoproteinemia due to protein malnutrition, the plasma lipid content has been found to be low rather than high (Feigl (1), Rosenthal and Patrzek (7), Man and Gildea (3)).

It appears that the sources of pathological variations in plasma lipids may remain obscure until the effects of different nutritional factors on lipemia have been ascertained under experimentally controlled conditions. As a step towards obtaining information on the nutritional factors, the present work was undertaken. The behavior of the plasma lipids in normal dogs has been studied over long periods under conditions of normal protein intake, and of protein intakes reduced so low that marked hypoproteinemia and gross edema resulted. The intakes of total calories, vitamins, and minerals, and other physiological factors, except protein intake, were kept as constant as possible.

Methods of Analysis

The total lipids, total cholesterol, and lipoid phosphorus in the heparinized plasma were determined by the gasometric methods of Kirk, Page, and Van Slyke (4).

Fractionation of the serum protein was accomplished by Howe's (2) method. Nitrogen was measured as ammonia after micro-Kjeldahl digestion; the ammonia was distilled in steam into N/70 hydrochloric acid and titrated back with N/70 sodium hydroxide. Serum albumin was calculated as 6.25 times the difference between nitrogen in the globulin-free filtrate and non-protein nitrogen.

Diet and Notes on Conditions of Animals

Diet—The composition of the low protein diet has been described previously (8). 100 gm. furnish 134 calories distributed as follows: protein 3.5, fat 53.0, carbohydrate 77.5 calories. The diet is offered at an approximate level of 75 calories per kilo of body weight. Dogs maintained on the diet are in negative nitrogen balance, show a progressive loss of albumin from the serum, and finally develop edema (8, 9). When the diet is supplemented by the addition of casein it has proved satisfactory for maintenance of health. The adequacy of the diet from the standpoint of vitamins has been discussed in another place (10).

Animals—Five dogs were studied during the development of hypoproteinemia and edema. Brief protocols of these animals follow.

Dog 4-87—A female police dog, aged 3 years, was started on the low protein diet supplemented by an adequate amount of casein on October 19, 1934. On November 5 the serum albumin level was 3.39 gm. per cent and the body weight was 17.1 kilos; at this time casein was removed from the diet and the period of protein deprivation begun. The appetite was excellent and there were no refusals of food until December 18. Subsequent anorexia was mild; refusals, amounting to more than half of the food offered, were not observed until 3 days before death. On February 21, 1935, the dog was still alert but had developed diarrhea. On the following morning it was found dead. Autopsy disclosed the presence of peritonitis. There were 4300 cc. of fluid in the peritoneal cavity.

The lowest body weight, 15.4 kilos, was observed on December 31. Subsequently (during the period covered in Table I) the weight increased progressively as ascites developed. On the day preceding death the weight was 20.7 kilos. On January 27 subcutaneous edema first appeared; thereafter it increased until both fore legs were moderately edematous and both hind legs exhibited massive edema. On February 4 samples of subcutaneous edema fluid and ascitic fluid contained, respectively, 0.31 and 0.12 gm. per cent of protein.

Dog 5-31—A male hound, aged 3 years, was subjected to alternate periods of adequate feeding and protein deprivation between December 17, 1934, and April 15, 1935. On April 15, when it had been on an adequate diet for 20 days, serum was taken for analysis and the low protein diet was started again. On May 26 edema of both ears was present and on the following day the legs were edematous. The edema increased until the experiment was discontinued on June 6. The appetite of this animal was at all times excellent.

Dog 5-69—A male police dog, aged 1 year, was given the low protein diet supplemented with casein on March 4, 1935. The period of protein deprivation was started when the casein was removed from the diet on April 1, when the dog weighed 16.2 kilos. There were no refusals of food until May 23. After this the appetite began to fail and on June 4 the diet was reduced to one-half the original amount; this level of feeding was maintained throughout the remainder of the experiment. Edema first appeared on May 29 and rapidly extended to involve all four legs. There was no ascites. General health was satisfactory until the experiment was discontinued on July 10. At this time the dog weighed 11.6 kilos.

Dog 5-70—A male police dog, aged 1.5 years, was given the casein-supplemented diet on March 18, 1935. The casein was removed and depletion of body protein started on April 1. At this time the serum albumin was 3.38 gm. per cent and the body weight was 16.7 kilos. At first the appetite was excellent but after May 26 there were daily refusals of a portion of the diet. On June 4 the diet was reduced to one-half the original allowance; this level of feeding was continued for the rest of the experiment. Edema appeared on May 29 and extended to involve all of the extremities. There was no ascites. Death occurred from inanition on July 7, with a body weight of 9.4 kilos.

Dog 4-83—A collie, aged 2 years, was maintained on the low protein diet from April 18, 1935, to July 16. The appetite and general health were good. This experiment was designed for other purposes and no attempt was made to continue the observations until edema resulted. The initial weight was 17.3 kilos; the final weight was 13.1 kilos.

DISCUSSION

The data in Table I show great variations in serum lipids from one dog to another. In order to estimate the effects of the dietary changes, it is therefore necessary to compare in the case of each dog the lipid values observed when it was on a normal diet with values on the low protein diet. From such comparisons

TABLE I

Lipids and Albumin in 100 Cc. of Plasma

Dog No.	Date	Total lipid carbon	Cholesterol, total	Lipoid phosphorus	Total lipids as carbon × 1.3	Total lipids as cholesterol	Total lipids as phosphatides*	Serum albumin	Edema	Preceding diet
	1935	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*	*per cent*	*gm.*		*days*
4-87	Jan. 3	200		3.7	260		33.4	1.71	None	60 Low protein
	" 21	205	35	6.8	266	13.1	59.8	1.54	Ascites	78 " "
	Feb. 6	214		5.4	278		45.6	1.17	Very marked ascites	94 " "
	" 18	221	52	4.7	287	18.1	38.4	1.12	" " "	106 " "
5-31	Jan. 10	195	47	3.0	253	18.6	27.9	2.18	None	20 Low protein
	Apr. 15	327	87	9.4	425	20.5	52.0	3.93	"	20 Normal diet
	May 10	269	105	9.0	350	30.0	60.2	2.60	"	22 Low protein
	" 28	232	66	7.9	301	21.9	61.7	1.76	Edema	40 " "
5-69	Mar. 28	444	104	12.0	577	18.1	49.1	3.39	None	24 Normal diet
	Apr. 18	706	233	23.5	918	25.5	60.0	2.65	"	17 Low protein
	May 17	946	376	31.2	1230	30.6	58.1	2.20	"	46 " "
	June 7	825	286	18.0	1073	26.6	39.4	1.56	Edema	67 " "
	July 1	568	220	16.2	738	29.8	51.6	1.24	"	91 " "
5-70	Mar. 28	640	142	18.7	832	17.1	52.9	3.38	None	10 Normal diet
	Apr. 18	750	195	20.9	975	20.0	50.0	2.41	"	18 Low protein
	May 17	825	326	26.7	1072	30.4	58.1	1.93	"	47 " "
	June 7	422	154	11.9	549	28.0	50.6	1.26	Edema	67 " "
	July 1	480	157	10.3	624	25.2	38.8	1.29	"	86 " " †

4-83	Apr. 16	469	121	14.7	610	19.8	56.6	3.50	None	Normal diet
	May 10	583	228	16.9	758	30.1	52.4	2.78	"	22 Low protein
	June 7	697	262	17.8	906	28.9	46.1	2.05	"	37 " "
	July 15	879	320	22.6	1143	28.0	46.5	1.66	"	67 " "
Control 1		540	170	18.0	702	24.2	60.2			Normal diet
" 2		663	194	18.2	862	22.5	49.7			" "
" 3		661	165	13.9	859	19.2	42.6			" "
" 4		731	143	23.2	950	15.0	57.6			" "
" 5		407	126	11.6	529	23.8	51.4			" "

* The phosphatides are calculated as 23.5 × lipoid phosphorus (Kirk, Page, and Van Slyke (4)).

† The dog died several days later.

the only conclusion apparently to be drawn is that the change from normal to low protein diet had no consistent effect on either the total lipid content or the proportions of the lipids in the forms of cholesterol and phosphatides.

Unfortunately control periods on a normal diet are lacking for Dog 4-87. The serum analyses are included, however, because they show progressive fall in albumin to the lowest level with maximal edema formation. These progressive changes were unaccompanied by any significant changes in serum lipids; on the contrary the latter remained unusually constant.

SUMMARY

Determinations of total serum lipid, total cholesterol, and lipoid phosphorus, and of the albumin fraction of the serum proteins, were made over periods of several months in five dogs. During the observations the diet was changed from one of adequate protein content to one very low in protein. Even when the low protein diet was continued so long that the serum albumin fell below 1.5 gm. per 100 cc. and gross edema occurred, the serum lipids were not consistently altered, either in their total concentration, or in the proportions of cholesterol and phosphatides in the lipid mixture.

From these results it appears improbable that protein malnutrition in itself has an important direct effect on the serum lipid content of dogs.

BIBLIOGRAPHY

1. Feigl, J., *Biochem. Z.*, **85**, 365 (1918).
2. Howe, P. E., *J. Biol. Chem.*, **49**, 109 (1921).
3. Man, E. B., and Gildea, E. F., *J. Clin. Inv.*, **15**, 203 (1936).
4. Kirk, E., Page, I. H., and Van Slyke, D. D., *J. Biol. Chem.*, **106**, 203 (1934).
5. Page, I. H., and Farr, L. E., *J. Clin. Inv.*, **15**, 181 (1936).
6. Page, I. H., Kirk, E., and Van Slyke, D. D., *J. Clin. Inv.*, **15**; 101 (1936).
7. Rosenthal, F., and Patrzek, F., *Klin. Woch.*, **56**, 793 (1919).
8. Weech, A. A., Goettsch, E., and Reeves, E. B., *J. Exp. Med.*, **61**, 299 (1935).
9. Weech, A. A., Goettsch, E., and Reeves, E. B., *J. Exp. Med.*, **61**, 717 (1935).
10. Weech, A. A., and Paige, B. H., *Am. J. Path.*, **13**, 249 (1937).

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DISTRIBUTION IN BODY FLUIDS AND EXCRETION OF INGESTED AMMONIUM CHLORIDE, POTASSIUM CHLORIDE, AND SODIUM CHLORIDE

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This paper presents observations made during the first hours after oral administration of single doses of the salts. The changes observed in the composition of serum and urine have been correlated with the now accepted views concerning the composition and volume of the intracellular and extracellular body fluids.

These views may be summarized in part as follows: The extracellular fraction of the body fluid has approximately the composition of an ultrafiltrate of plasma, and in man contains most of the sodium and practically all of the chloride of the organism. The tissue cell membranes are ordinarily impermeable to the ions, Na^+, Mg^{++}, Cl^-, HPO_4^{--}, SO_4^{--}, and SCN^-, and to sucrose; consequently, after an intravenous injection of a non-isotonic solution of one of these substances, a new equilibrium is reached between extra- and intracellular concentrations, not through exchange of these solutes, but through a movement of water (Peters, 1935; Lavietes, Bourdillon and Klinghoffer, 1936; Bourdillon and Lavietes, 1936).

Experimental procedures. The same subject (J. B.) male, in good health, and weighing approximately 65 kilos, was used for all experiments, which were performed in the post-absorptive state. No food was taken during the procedure: water was administered with the salt in sufficient amounts to avoid nausea; some water was absorbed later in moderate amounts during the experiments, as recorded in the tables. No catharsis resulted.

The chemical procedures used were as follows (Peters and Van Slyke, 1932): CO_2 in serum and urine after Van Slyke and Neill; Cl in serum after Patterson, and in urine after Volhard-Harvey for experiments 1, 2 and 3; Cl in serum and urine after Van Slyke and Sendroy for the other experiments; sodium in serum and urine after Butler and Tuthill; potassium in urine after Shohl and Bennett for experiments 1, 2 and 3; potassium in serum and urine after Kramer and Gittleman for the other experiments; ammonia in urine after Van Slyke and Cullen; urea in urine, with urease, after Van Slyke. Serum total base was determined after Hald (1933). The interstitial fluid volume was determined with CNS, as described previously (Lavietes, Bourdillon and Klinghoffer, 1936).

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[1] Experiments 1, 2 and 3 reported in this paper were made in the Department of Internal Medicine, Yale University.

TABLE 1

Effect of NH_4Cl perorally administered

TIME	SERUM				INTERSTITIAL FLUID VOLUME	TIME	URINE						REMARKS
	CO_2	Cl	Total base	Urea N			Cl	Na	K	NH_3	Urea N	Volume	
	m.Eq. per liter	*m.Eq. per liter*	*m.Eq. per liter*	*m.Eq. per liter**	*liters*		*m.Eq. per hour*	*m.Eq. per hour*	*m.Eq. per hour*	*m.Eq. per hour*	*m.Eq.* per hour*	*cc. per hour*	
9:15	28.8	99.9	149.1		19.2	8:00– 9:15				0.6†		40	*Exp. 1.* 9:15–9:40 a.m.: 182 m.Eq. NH_4Cl + 700 cc. 1:00 p.m. 150 cc. coffee + light lunch
12:15	21.3	106.8	150.4		18.6	9:15–12:15	17.7	11.0	3.8	4.2†		138	
						12:15– 2:15	31.5	20.0	6.5	4.8†		158	
7:20	29.6	100.4			16.4								*Exp. 2.* 7:20–7:50 a.m.: 182 m.Eq. NH_4Cl + 600 cc. water 10:30, 150 cc. coffee
10:30	23.1	106.9			16.2	7:20–10:30	15.3	7.6	5.0	4.5†		175	
1:20	23.6	105.9			15.9	10:30– 1:20	21.7	10.8	8.8	3.9†		88	
8:15	27.0	100.5	144.5		18.0								*Exp. 3.* 8:15–8:45 a.m.: 182 m.Eq. NH_4Cl + 600 cc. water 11:30, 150 cc. coffee
11:15	23.3	106.4	145.2		17.9	8:15–11:15	16.2	9.7	3.5	3.7†		70	
						11:15–12:45	26.6	15.6	8.3	4.2†		333	
8:40	30.6	101.8		7.5		7:05– 8:40				1.8		30	*Exp. 4.* 8:45–9:35 a.m.: 187 m.Eq. NH_4Cl + 800 cc. water
11:30	23.2	108.4		10.1		8:40–11:30	15.5			5.2	44.0	247	
12:50	23.5	107.8		9.7		11:30–12:50	14.0			4.1	42.7	131	

* Expressed as m.Eq. of NH_4.

† These determinations were made by Dr. P. H. Lavietes.

DISCUSSION OF RESULTS. *Effects of NH_4Cl ingestion. Excretion.* That ammonia excretion was accelerated within the first three hours is shown by table 1. It is well known that after repeated NH_4Cl administration over periods of many hours or one or more days a great increase of ammonia output can occur (Gamble, Blackfan and Hamilton, 1925). It has been shown in this laboratory in experiments on dogs with explanted kidneys (Van Slyke, Rhoads, Hiller and Alving, unpublished) that ammonia absorbed as NH_4Cl is so quickly changed to urea, that there is but very slight increase in the arterial blood ammonia, and that the ammonia excreted by the kidney is not at all the same ammonia absorbed, but is freshly formed in the kidney itself by the mechanism first discovered by Nash and Benedict (1921). If the same is true for man, it follows that the kidney responds to the acidosis with increased ammonia formation rather more quickly than had been supposed.

The water output during the experiments was approximately equal to the intake, so that there was no significant change in the fluid content of the body.

The rate of Cl excretion was such that in three hours after ingestion one quarter, or somewhat more, of the administered Cl was excreted.

Serum chloride. In table 2 the fourth column gives the Cl concentration increments theoretically calculated as $\frac{\text{Retained Cl}}{\text{Liters interstitial fluid}}$. The assumptions on which the calculation is based are: 1. In the first 3 hours all the ingested Cl was absorbed from the gastrointestinal tract, so that the Cl retained in the body proper could be calculated as ingested Cl minus excreted Cl. 2. The retained Cl was distributed uniformly in the serum and other interstitial fluids. 3. None of the retained Cl penetrated the tissue cells (that entering the red blood cells and concentrated in the kidney is neglected). 4. The interstitial fluid volume was measured with sufficient accuracy by the sulfocyanate method.

TABLE 2

Calculated and observed changes in serum Cl and CO_2 after NH_4Cl administration

EXPERIMENT NUMBER	*a* RETAINED Cl CALCULATED AS INTAKE − OUTPUT	*b* INTERSTITIAL FLUID VOLUME	$c = \frac{a}{b}$ CALCULATED + Δ Cl	OBSERVED − Δ CO_2	OBSERVED + Δ Cl	OBSERVED + Δ UREA
	m.Eq.	*liters*	*m.Eq. per liter*	*m.Eq. per liter*	*m.Eq. per liter*	*m.Eq. per liter*
1	129	19	6.8	7.5	6.9	
2	134	16	8.4	6.5	6.5	
	72	16	4.5	6.0	6.5	
3	133	18	7.4	3.7	5.9	
4	143	17*	8.4	7.4	6.6	2.6
	124	17*	7.3	7.1	6.0	2.2

* Assumed value.

The agreement between the fourth and sixth columns in table 1 indicates that the results are consistent with these assumptions.

Serum urea. It has been repeatedly shown (Peters and Van Slyke, 1931) that when NH_4Cl is ingested the ammonia is transformed into urea, and that urea in the higher animals diffuses freely into both intra- and extracellular fluids, so that (except for the kidney) it is found nearly everywhere in the body in approximately the same concentration per unit of water present (Peters, 1935). Granting these premises, and that the interstitial fluids comprise about ⅓ of the body fluids, one would expect that, after NH_4Cl ingestion, increase in urea concentration in all the fluids would be about ⅓ the increase in Cl concentration, since the latter is confined to the interstitial fluids. Comparison of the last two columns of table 2 indicates that the serum urea increment was in fact about ⅓ the Cl increment.

Serum CO_2. The agreement between the fifth and sixth columns of table 2 confirms Gamble and Ross (1923) in showing that the serum CO_2 decrease caused by NH_4Cl ingestion approximates the Cl increase. Since CO_2 changes within the limits noted are approximately (ca. 95 per cent) bicarbonate changes, near equality of Cl and CO_2 changes would be expected if the HCl, liberated by change of the NH_3 into urea, decomposes an equivalent of bicarbonate in the interstitial fluids.

Effects of KCl ingestion. Excretion. The striking fact evident in the right hand side of table 3 is that there was not only an increase in the excretion rates of K and Cl, which was expected; there were also marked, though short-lived, increases in the renal outputs of Na and CO_2 (the CO_2 representing in large part HCO_3). The outpouring of the latter ions began quickly; it also ended quickly, so that in the second urine, passed later than 2 hours after ingestion of the KCl, the rates of Na and HCO_3 excretion had fallen most of the way to their original levels.

The immediate effect of the KCl absorption was therefore to cause an excretion of all four of the chief plasma ions, Na, Cl, HCO_3 and K. In its apparent haste to get the total electrolyte concentration of the plasma down to normal, the kidney seemed to lose some of its usual discrimination; instead of eliminating only the K and Cl that had invaded the body, it also excreted Na and HCO_3 during the period immediately following the ingestion.

Serum electrolytes. The serum chloride increase in the experiments of table 3 (first two hours after KCl ingestion) was approximately what one would calculate on the assumption that the retained Cl was distributed uniformly in the plasma and other interstitial fluids (as after NH_4Cl ingestion) and also that there was no dilution of interstitial fluid. The serum K increase, however, was only about one-half as great. There was at the same time a decrease in serum CO_2.

Possible mechanism of the electrolyte changes following KCl ingestion. The sequence of events may be the following:

1. Leaving the gastrointestinal tract, K and Cl diffuse together into the blood and extracellular water. 2. The Cl stays in the extracellular fluids and does not penetrate the cells (except the erythrocytes) but the K distributes itself to the intracellular fluids as well. In consequence of

the wider distribution of the absorbed K, the increment of K concentration in the plasma is less than the Cl increment. 3. The K in diffusing into the cells must be accompanied by some anion. The tissue cells appear to be impermeable to Cl but not to HCO_3 (Fenn, 1936). Hence it appears

TABLE 3

Effect of KCl perorally administered

TIME	SERUM CO_2	SERUM Cl	SERUM K	INTERSTITIAL FLUID VOLUME	TIME	URINE CO_2	URINE Cl	URINE Na	URINE K	URINE Urea N	URINE Volume	REMARKS
	m.Eq. per liter	*m.Eq. per liter*	*m.Eq. per liter*	*liters*		*m.Eq. per hour*	*m.Eq. per hour*	*m.Eq. per hour*	*m.Eq. per hour*	*mgm. per hour*	*cc. per hour*	
10:20	28.7	104.0	4.2	16.6	8:15–10:20	0.3	6.0	2.0			39	*Exp. 5.* 10:20–10:30: 161 m.Eq. KCl + 350 cc. water
12:05	26.2	108.0	6.4	16.5	10:20–12:05	8.6	23.4	12.0	23.6*		120	
					12:05– 1:20	2.0	24.8	4.8	25.7*		88	
10:20	30.7	102.6	4.7		9:15–10:20	0.7	5.5	2.3	3.6		50	*Exp. 6.* 10:25–10:35: 154 m.Eq. KCl + 350 cc. water 12:30; 250 cc. water + 15 gm. glucose
12:15	27.2	108.4	7.5		10:20–12:15	8.7	28.5	14.1	26.0	455	157	
					12:15– 1:55	2.0	28.4	5.5	24.8	387	96	
					1:55– 3:15	0.9	17.8	4.6	12.4		60	
8:30	30.0	103.8	5.6		(8:35–10:00)	(11.4)					(335)	*Exp. 7.* 8:35–9.05: 161 m.Eq. KCl + 500 cc. water 11:10; 150 cc. water + 15 gm. glucose
11:00	26.8	108.2	8.0		8:35–11:00	9.0	40.6	22.1	31.0		267	
12:05	26.7	106.8	6.5		11:00–12:05	0.1	24.9	3.7	23.5		83	

* As urine was not ashed, these figures should be slightly too high.

TABLE 4

Calculated and observed changes in serum Cl, K, and HCO_3 after KCl ingestion. First period of 2 to 3 hours after ingestion in each experiment

EXPERIMENT NUMBER	*a* RETAINED K AND Cl CALCULATED AS INTAKE—OUTPUT		*b* INTERSTITIAL FLUID VOLUME	$c = \frac{a}{b}$ CALCULATED		OBSERVED		CALCULATED* $-\Delta HCO_3$ FROM URINE HCO_3 OUTPUT ALONE	OBSERVED	
	Cl	K		$+\Delta Cl$	$+\Delta K$	$+\Delta Cl$	$+\Delta K$		$-\Delta HCO_3$	$\Delta Cl - \Delta K$
	m.Eq.	*m.Eq.*	*liters*	*m.Eq. per liter*	*m.Eq. per liter*	*m.Eq. per liter*	*m.Eq. per liter*	*m.Eq. per liter*	*m.Eq. per liter*	*m.Eq. per liter*
5	120	120	16.5	7.3	7.3	4.0‡	2.2	0.9	2.5	1.8
6	99	104	(17)**	5.8	6.1	5.8	2.8	1.0	3.5	3.0
7	63	86	(17)**	3.7	5.1	4.4	2.4	1.3	3.2	2.0

* HCO_3 in this column is calculated as mM. CO_2.

** Assumed interstitial fluid volume, from average on this subject.

‡ The first period in experiment 5 covered only 1¾ hours after KCl ingestion. The fact that observed serum Δ Cl was lower than calculated indicates that absorption in this period was probably not completed.

probable that the K which passes into the tissue cells is accompanied by an equivalent of HCO_3.

If these were the only events influencing the three ions in the plasma, the plasma concentration changes, indicated as Δ values, could be expressed by the equation:

$$\Delta Cl - \Delta K = -\Delta HCO_3 \tag{1}$$

The left hand member, multiplied by the interstitial fluid volume, would represent the amount of absorbed K that has passed into the tissue cells, while the right hand member similarly multiplied would represent the HCO_3 accompanying the K into the cells.

If the observed values of table 4 are applied to the equation, the values for $-\Delta HCO_3$ calculated from the K and Cl changes are found to be from 0.5 to 1.2 m.Eq. smaller than $\Delta Cl - \Delta K$, indicating that, if only one equivalent of HCO_3 accompanied the K into the cells, some additional CO_2 left the interstitial fluids by another channel. This channel is indicated by the urinary CO_2 values of table 3 to be the kidneys. The amounts of CO_2 excreted in the second periods of experiments 5, 6 and 7 vary from 15 to 21 millimoles, which is from 0.9 to 1.3 millimoles per liter of interstitial fluid. Allowing for the fact that part of the CO_2 in the urine was free carbonic acid, only part in the form of HCO_3, the HCO_3 excretions appear to be close to the amount, 0.5 to 1.2 m.Eq. per liter interstitial fluid, which one must add to the right hand member of equation (1) in order to make the equation balance.

The above explanation is based on the assumptions that the tissue cells are permeable to K, and either to HCO_3 anions, or to CO_2 capable of conversion to HCO_3 within the cells. For these assumptions with regard to both HCO_3 and K the literature offers evidence.

With regard to HCO_3 Fenn (1936) has found that the muscle cells of the frog contain about 7 m.Eq. per kilo, and that carbonic acid added to blood in which frog muscle was suspended penetrated into the muscle cells.

With regard to potassium Fenn (1936) summarizes data showing that muscle cells are permeable to it, and that there is in the animal equilibrium between K in the muscle cells and K in the plasma, such that K passes back and forth between cells and plasma in response to changes in plasma K concentration and pH. In experiments where carbonic acid was added to frog muscle in frog blood, Fenn found that K was "driven from the blood into the muscle to neutralize the acid inside the muscle." Furthermore, evidence for the general penetration of retained K into the tissue cells is furnished by Norn (1929) in an injection experiment with a nephrectomized rabbit. His figures showed that the increase in serum K concentration was so low that the great part of the injected K must have left the extracellular fluids, the only apparent path of exit being into the tissue cells. With Dr. I. H. Page we have confirmed this finding in an experiment as follows:

A 3000 gram rabbit was narcotized with amytal, and the ureters were ligated. Then 8 millimoles of KCl were injected intravenously with 50 cc. of water, during a period of 70 minutes. The K concentration of the serum rose from a pre-injection value of 2.4 m.Eq. to 6.6 m.Eq. per liter. To limit the rise to the observed 4.2 m.Eq., the injected 8 m.Eq. of K would have to be distributed uniformly in 1.9 liters of fluid, which corresponds closely to the total water content of a 3000 gram rabbit.

Concerning the hypothesis that retention of HCl is followed by migration of $KHCO_3$ into the tissue cells, it therefore appears from the above discussion that the migration of K may be assumed with fair certainty, and that migration of the HCO_3 is a possibility consistent with what is at present known. To make the evidence stronger, it would be desirable to perform with $KHCO_3$ animal experiments like that above outlined with injected KCl, and to analyze both plasma and tissues for CO_2. The necessity for special technique for accurate CO_2 analyses in tissues has prevented such experiments in connection with the work here reported.

TABLE 5

Effect of NaCl perorally administered

TIME	SERUM			TIME	URINE					REMARKS
	CO_2	Cl	Na		CO_2	Cl	Na	K	Volume	
	m.Eq. per liter	*m.Eq. per liter*	*m.Eq. per liter*		*m.Eq. per hour*	*m.Eq. per hour*	*m.Eq. per hour*	*m.Eq. per hour*	*cc. per hour*	
9:10	29.1	102.8	142.7							*Exp. 8.* 9:15–9:35: 210 m. Eq. NaCl + 400 cc. water 12:05: 400 cc. water + 20 gm. glucose
12:05	28.6	108.4	147.1	9:10–12:05	0.2	14.9	7.3	10.3	53	
				12:05– 1:15	0.1	12.7	5.3	9.3	43	

Without tissue bicarbonate data, the possibility remains that the decrease in serum bicarbonate might have been due to acid decomposition of HCO_3. Such an occurrence would follow only if the KCl retention accelerated acid production by the organism.

If our interpretation of the results is correct, potassium chloride administration must increase the HCO_3, and in consequence the pH, within the tissue cells, as it does in the urine, while at the same time it decreases HCO_3 and pH in the extracellular fluids. There would result from KCl retention the curious phenomenon of a shift toward acidosis in the extracellular fluid, and toward alkalosis within the cells, whereas the effect of NH_4Cl would be only acidosis in the extracellular fluid.

Effects of sodium chloride ingestion. Finally, in table 5, a similar experiment with NaCl ingestion is given for comparison. This time a marked water retention is evident. There is no increase of bicarbonate excretion, nor appreciable change in serum CO_2. Cl is excreted only in part as NaCl; part passes out as KCl. The Na and Cl increases in serum each approximate 5 m.Eq.

If the interstitial fluid had remained constant at 17 liters the Cl and Na retained would have raised their concentrations in plasma by 10.6 m.Eq. However, in this experiment interstitial fluid measurements indicated an increase of 700 cc., which was the volume of water retained. This would have decreased the Cl and Na concentrations of the second serum sample by 4 per cent, or 4 and 6 m.Eq. respectively. It appears that the 700 cc. of water retained remained in the interstitial fluids, and prevented about half of the increase in Na and Cl concentration in those fluids that would otherwise have been caused by the NaCl retention.

SUMMARY

Single large doses of NH_4Cl, KCl and NaCl were administered by mouth to a human subject, and the changes in serum electrolytes, interstitial fluid volume, and electrolyte excretion were observed for several hours. These changes suggest the following conclusions:

1. The increment in serum Cl concentration following the administration of each salt approximates that estimated by assuming that the retained Cl diffuses into the interstitial fluid only, and does not enter the tissue cells.

2. The increment in serum urea concentration following NH_4Cl administration approximates that estimated by assuming that the retained N is entirely changed to urea, which diffuses into the total body fluids, both intra- and extracellular.

3. The increment in serum K concentration after KCl administration approximates that estimated by assuming that the retained K, like urea, diffuses into the total body fluids.

4. As regards the behavior of serum bicarbonate, it appears that:

After NH_4Cl administration it was reduced by acid decomposition.

After KCl administration, it was also reduced. About $\frac{1}{3}$ of the fall in serum HCO_3 was accounted for by excretion of bicarbonate in the urine. For the other $\frac{2}{3}$ the probable exit was by way of diffusion as $KHCO_3$ into the tissue cells, although the possibility of HCO_3 decomposition by accelerated acid formation in the body is not excluded.

After NaCl administration, serum bicarbonate was not appreciably affected.

REFERENCES

BOURDILLON, J. AND P. H. LAVIETES. J. Clin. Investigation **15**: 301, 1936.

FENN, W. O. Physiol. Reviews **16**: 450, 1936.

GAMBLE, J. L., K. D. BLACKFAN AND B. HAMILTON. J. Clin. Investigation **1**: 359, 1925.

GAMBLE, J. L. AND G. S. ROSS. Am. J. Dis. Child. **25**: 470, 1923.

HALD, P. M. J. Biol. Chem. **103**: 471, 1933.

LAVIETES, P. H., J. BOURDILLON AND K. A. KLINGHOFFER. J. Clin. Investigation **15**: 261, 1936.

NASH, T. P., JR. AND S. R. BENEDICT. J. Biol. Chem. **48**: 463, 1921.

NORN, M. Skand. Arch. f. Physiol. **55**: 211, 1929.

PETERS, J. P. Body water. Charles C. Thomas, Springfield, Illinois, 1935.

PETERS, J. P. AND D. D. VAN SLYKE. Quantitative clinical chemistry. Vol. I (1931); Vol. II (1932) Williams & Wilkins, Baltimore.

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THE EFFECT OF DIET ON THE SUSCEPTIBILITY OF THE CANINE HEMATOPOIETIC FUNCTION TO DAMAGE BY AMIDOPYRINE

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PLATE 10

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The association of amidopyrine administration with the occurrence of acute agranulocytosis in human beings has been pointed out in a number of communications (1–4). However, attempts to confirm by experimentation the hypothesis of a toxic effect of amidopyrine on hematopoiesis have failed signally. Although in rare instances a decrease in the number of circulating granulocytes has been reported to follow the administration of amidopyrine to animals, such results have been so irregular as to throw serious doubt on the rôle of the drug as an inhibitor of blood formation.

Miller (5) and Climenko (6) have published evidence that in animals fed amidopyrine in solution, histological examination of the bone marrow discloses definite pathological changes even though no marked decrease in the numbers of granulocytes in the circulating blood is present. Miller and Rhoads (7), in a study of the hematological changes associated with acute black tongue in dogs, reported that the acute stomatitis was occasionally associated with a decreased number of granulocytes in the peripheral blood. Histological studies of bone marrow removed at the height of the granulopenia revealed a relative decrease of mature hematopoietic elements. The change observed presented certain similarities to the pathological alteration of the bone marrow in acute granulopenia of man.

In the case of acute stomatitis and granulopenia of the dog, the etiology is apparent; it is the feeding of a particular diet. In acute granulopenia of man the pathological changes are in certain respects similar to those of the dog, but the cause is obscure although amidopyrine administration appears to play some part. It seemed possible then, that a faulty dietary might well be one factor at least which increased the susceptibility to the toxic effect of amidopyrine. To substantiate this hypothesis experiment should prove that a dose of amidopyrine which is ineffective alone will cause a well defined disturbance of hematopoiesis when it is added to a diet which is not by itself causative of symptoms. Such experiments have been performed, but paradoxically, the resulting disturbance of the circulating blood cells manifested itself in the erythrocytes rather than in the leukocytes. The results are reported in this communication.

The experiments dealing with the effects of aromatic compounds on hematopoiesis have been reviewed recently in detail by Hamilton (8) and by Kracke (9) and need no discussion here. It suffices to state that the picture of progressive anemia, entirely similar to aplastic anemia in man, has not been encountered. Sudden and severe decreases in number of white cells have been the rule. This last fact led Kracke (1) to suggest that the rôle of amidopyrine in granulopenia was one similar to that of benzol in the blood dyscrasias which are due to that chemical.

The part played by diet in controlling the susceptibility of individuals to poisoning by aromatic compounds was suggested by studies of trinitrotoluol poisoning in Great Britain during the World War (10). Furthermore, Biberfeld (11) had observed that an inadequate dietary rendered rabbits susceptible to poisoning by amounts of methol which were non-toxic to animals fed a normal diet.

Methods

The animals employed were mongrel dogs of about 7 kilos in average weight. They were kept under standard conditions in individual cages with bedding of wood shavings.

The so called normal diet was one which is fed as routine; and empirically is known to be capable of maintaining dogs in good health over a period of several years. It is a mixture of cooked beef, bread, and dog biscuit. The black tongue diet was that described by Goldberger (12). It is known to cause acute black tongue when fed, without supplement, for a period of from 5 to 10 weeks. In an extensive study the feeding of this diet has never been known to cause symptoms in previously normal dogs after a shorter interval. The diet was composed of the following ingredients.

Articles of diet	Quantity	Nutrients		
		Protein	Fat	Carbohydrate
	gm.	*gm.*	*gm.*	*gm.*
Corn meal	400	33.6	18.8	296
California black-eyed peas	50	10.7	0.7	30.4
Casein (purified)	60	52.0		
Cane sugar	32			32.0
Cottonseed oil	15		15.0	
Cod liver oil	30		30.0	
Sodium chloride	3			
Calcium carbonate	10			
Total nutrients		96.3	64.5	358.4
Nutrients per 1000 calories		40.1	26.9	149.3

The corn meal, peas, and casein were mixed and cooked for 2 hours in a steam cooker. The remaining ingredients were then added and thoroughly mixed. The dogs were fed daily and were allowed to eat as much as they chose.

Blood was taken from the jugular vein in a standard amount of potassium oxalate for routine examinations. Determinations of the number of erythrocytes and leukocytes were made in standard pipettes and counting chambers. The hemoglobin was estimated by the Sahli method, employing a glass standard. The Sahli tubes were carefully calibrated and checked at frequent intervals by the O_2-combining capacity method of Van Slyke (13).

The amidopyrine used was lot 1,433, manufactured by the H. A. Metz Laboratories, New York. It was administered by stomach tube in a 5 per cent solution in water. Mild heating was required to effect a solution of that strength.

General Results

The effect of the administration of 0.5 gm. of amidopyrine daily was tested on two groups of dogs. One group of 12 was fed the Goldberger black tongue diet and the other group of 3 received the normal diet. In the first group (Table I), severe ulcerative stomatitis and profound anemia appeared between the 2nd and 9th weeks of treatment in 9. Three dogs, Nos. 2, 11, and 12, failed to develop severe anemia. Anorexia, loss of weight, and diarrhea were marked features. No striking granulopenia was observed. The bone marrows of the animals which died revealed a suppression of hematopoietic maturation. In the control group of animals (Table II) which were subjected to the same medication but were fed the normal diet, no symptoms resulted. The experiment was repeated, employing larger doses of amidopyrine (Tables III and IV). Both stomatitis and anemia were severe in the animals fed the black tongue diet, but in those fed the normal diet only a mild anemia resulted. No distinct granulopenia occurred at any time in any experimental animal although histological study of the bone marrows of the animals which died showed a well defined interference with maturation of both red and white cells.

Anemia

The fall of erythrocyte levels was rapid in those animals in which anemia occurred. In the group fed amidopyrine plus the diet producing black tongue, the anemia was profound, the erythrocyte count in some instances reaching 50 per cent or less of the original level. In general, the fall of hemoglobin levels paralleled the fall in erythrocytes, giving a color index which is approximately normal for the dog. *Marked variation in size and shape of erythrocytes was a feature at the height of the anemia. Reticulocyte counts were not done. There was no increase in the bilirubin content of the serum as indicated by* the icterus index.

Stomatitis

The stomatitis appearing in those dogs fed the black tongue diet supplemented by amidopyrine was somewhat similar to that seen when the diet alone was fed for a longer period of time. The first manifestation was a marked dusky red coloration of the entire oral and pharyngeal mucous membrane. After a short time, small, discrete ulcerations appeared, most frequently just anterior to the pharyngeal pillars. These showed a base covered by yellow necrotic material and a raised, injected margin. At this stage, salivation was severe. In some instances the ulceration remained localized, and in others it progressed until multiple, coalescent lesions were present and were covered by a membrane of necrotic material. The loss of tissue was more severe, though more localized, than that seen in most instances of black tongue due to feeding alone.

Pathological Changes of the Bone Marrow

In Figs. 1 and 2 are shown low and high power photomicrographs of the bone marrow of dog 3, which developed severe anemia and died following the combination of feeding the black tongue diet and the daily administration of 0.5 gm. of amidopyrine. Similar changes were seen in other animals which died.

The normal arrangement of femoral bone marrow in the dog is almost completely obliterated. Instead of well defined islands of orderly hematopoiesis, the cells are scattered irregularly throughout the marrow tissue (compare with Fig. 3 which shows normal canine, femoral marrow). There is a striking suppression of maturation, particularly as concerns the cells of the erythropoietic series. Normoblasts and late erythroblasts are almost completely absent. A very definite increase in young cells has taken place as shown by the presence of many undifferentiated forms which vary somewhat in size. The most common one is a small, round cell with a small amount of basophilic cytoplasm and a deeply staining nucleus containing a very large amount of chromatin. This is held to be the primitive erythropoietic cell of Sabin (14). Various modifications of the primitive cell are present; some have more cytoplasm and a looser arrangement of nuclear chromatin. All of the stages in development up to that of the erythroblast are represented but only a few examples of more mature red cells are at hand.

An interference with the maturation of the granulocytes is less marked but is definite, in spite of the absence of granulopenia in the circulating blood. Granular leukocytes and their precursors are present in reduced numbers in the marrow. Furthermore, they show well defined evidence of damage as indicated by pyknosis of nuclei, abnormal size and shape, and irregular staining. No interference with the megakaryocytes can be made out.

Experiment 1. Table I. Text-Fig. 1

In this experiment, 12 dogs fed the black tongue diet received 0.5 gm. of amidopyrine daily. The results can best be seen by examination of the protocol of an animal which is typical of the series. It should be noted that in this instance the oral lesions appeared in less than 10 days after the experimental diet feeding was begun, whereas such changes have never been observed in this laboratory from diet feeding alone before the 5th week.

Dog 4.—

5-31-34. Black tongue diet feeding begun. Amidopyrine administration 0.5 gm. daily begun.

6-12-34. Multiple small shallow punched out ulcers have appeared on the buccal mucosa. These range from 0.2-0.5 cm. in diameter and show a yellow base with a reddened margin. There is active salivation. The whole oral and pharyngeal mucous membrane is a deep and dusky red. There is fairly active diarrhea. The animal refuses food.

TABLE I

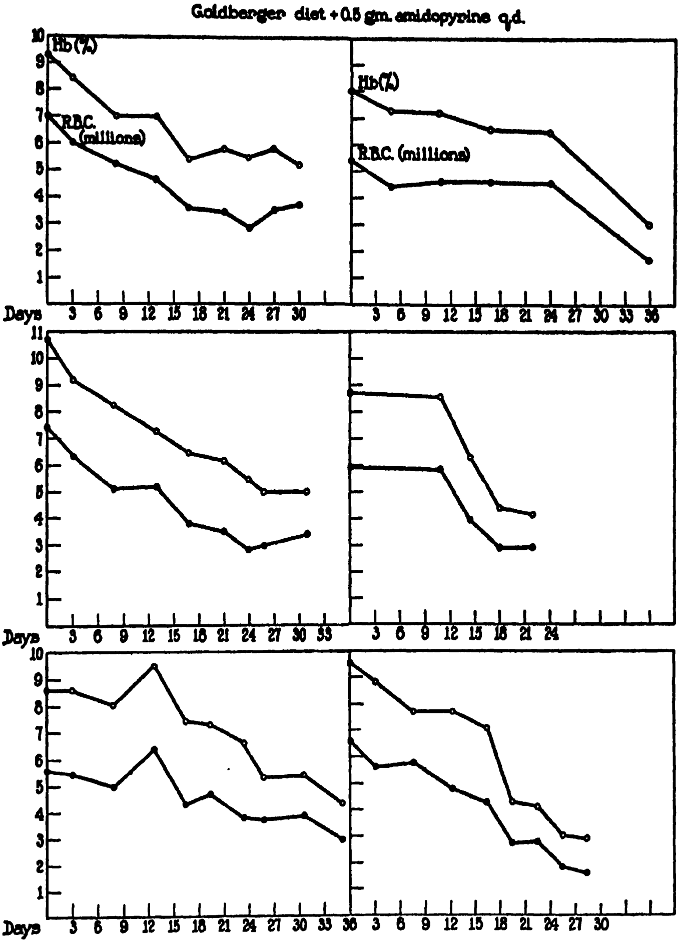
High and Low Blood Levels in Dogs Receiving Goldberger Diet and Amidopyrine, 0.5 Gm. Daily

Dog No.	Before amidopyrine		Time on amidopyrine	Time on diet	After amidopyrine		Remarks
	R.B.C. per c.mm.	Hb			R.B.C. per c.mm.	Hb	
	millions	*per cent*	*days*	*days*	*millions*	*per cent*	
1	560	62	8	8	249	39	Amidopyrine discontinued. Recovered
2	576	81	12	12	390	70	Died
3	672	111	29	29	175	30	"
4	669	102	33	33	356	62	"
5	558	86	35	55	293	44	Amidopyrine discontinued. Recovered
6	747	107	31	51	271	57	" "
7	704	93	30	50	288	55	" "
8	660	95	28	48	168	29	" "
9	589	87	19	33	291	41	" "
10	539	80	41	41	164	29	" "
11	459	90	57	57	399	73	—
12	783	103	30	40	527	92	—

6-16-34. The ulcerated areas which have shown little change since 6-12 have now extended and show a tendency to coalesce. They are much deeper and show more necrotic tissue at the border.

6-20-34. The buccal and pharyngeal mucous membrane is almost replaced by an extensive coalescent, deep ulceration with gangrene of the submucous tissues. Salivation is very active and the animal appears ill. Diarrhea has been present every day. There is marked loss of weight, complete anorexia, and progressive weakness. Amidopyrine is discontinued. From this time on there is a distinct tendency of the ulcerated areas to heal. Weakness and anorexia persist, however.

7-6-34.. After a progressive increase in weakness the animal is found dead.



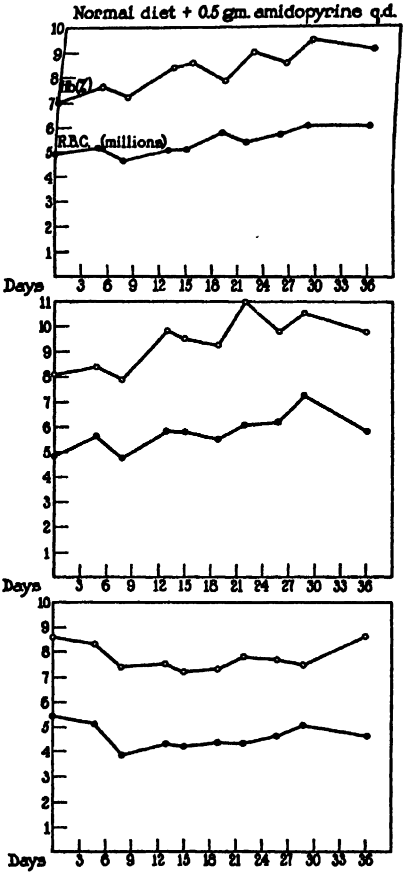
TEXT-FIG. 1

Autopsy.—An extensive gangrenous stomatitis is present. The marrow of the femur is a reddish yellow in color. No other gross lesions of the organs are seen. The microscopical changes are described under pathological alterations.

TABLE II

High and Low Blood Levels in Dogs Receiving Normal Diet and Amidopyrine, 0.5 Gm. Daily

Dog No.	Before amidopyrine		Time on amidopyrine	After amidopyrine		Remarks
	R.B.C. per c. mm.	Hb		R.B.C. per c. mm.	Hb	
	millions	*per cent*	*days*	*millions*	*per cent*	
1	644	86	36	468	86	Well
2	492	70	36	608	91	"
3	487	81	36	586	98	"



TEXT-FIG. 2

Experiment 2. Table II. Text-Fig. 2

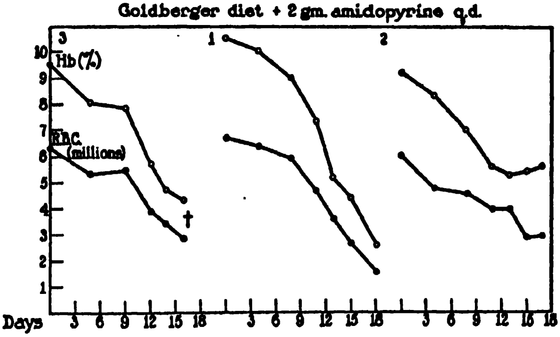
This experiment served as a control on Experiment 1. 3 dogs fed the normal diet were given 0.5 gm. of amidopyrine daily. The two experiments were performed in parallel and the conditions differed in no detail other than the diet.

In no instance did either mouth lesions or blood dyscrasia occur in this group of animals. They remained in excellent health throughout the period of observation.

TABLE III

High and Low Blood Levels in Dogs Receiving 2 Gm. Amidopyrine Daily and Goldberger Diet

Dog No.	Before amidopyrine		Time on amidopyrine	After amidopyrine		Remarks
	R.B.C. per c. mm.	Hb		R.B.C. per c. mm.	Hb	
	millions	*per cent*	*days*	*millions*	*per cent*	
1	6.63	105	19	1.55	26	Amidopyrine discontinued. Recovered
2	6.00	92	19	2.88	54	" "
3	6.29	95	16	2.89	43	Died after convulsion



TEXT-FIG. 3

Experiment 3. Table III. Text-Fig. 3

Three dogs were fed the black tongue diet and received 2 gm. daily of amidopyrine. The results of the treatment are presented in Table III and in Text-fig. 3. All of the animals developed a severe grade of anemia and one died. In the remaining 2, treatment was discontinued and recovery ensued. One striking discrepancy between this experiment and Experiment 1, in which only 0.5 gm. of amidopyrine was administered daily, was observed. The animals receiving a smaller amount of the drug all showed ulceration of the oral mucosa, whereas those given a larger amount did not manifest that change. No explanation is at hand for this difference.

Dog 3.—

4-23-34. Black tongue diet administration begun.

5-9-34. Amidopyrine, 2 gm. daily begun.

5-16-34. A tonic and clonic convulsion occurs lasting several minutes following the amidopyrine administration. This is followed by a period of marked weakness of the extremities.

5-18-34. Severe salivation follows the amidopyrine administration. No more convulsions have been observed.

5-26-34. The animal has a severe anemia and is weak and pale. Amidopyrine administration is again followed by a convulsion. Later the dog is found dead.

Autopsy.—No abnormality of the organs except the bone marrow is found other than a very marked degree of pallor. The bone marrow appears redder and more cellular than normal to gross examination.

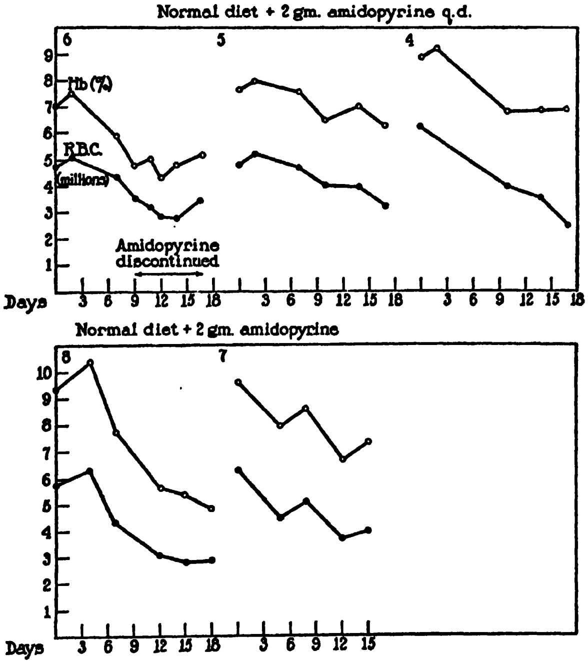
Experiment 4. Table IV. Text-Fig. 4

This experiment served as a control on Experiment 3. 5 animals were fed the normal diet and at the same time given 2 gm. of amidopyrine daily. The results are shown in Table IV. In contrast to the animals of Experiment 2, which received the same diet but only 0.5 gm. of amidopyrine daily, those given the larger amount of the drug did evince a definite fall in erythrocyte values. At no time, however, were mouth lesions or evidence of gastro-intestinal tract involvement, such as diarrhea, present. The animals continued to eat well and remained in relatively good health. The grade of anemia encountered in this group was not as severe as that seen in the animals fed the black tongue diet. In further experiments of this type similar results have been encountered. Whereas some depression of erythrocyte levels can be caused by the prolonged administration of large amounts of amidopyrine, it is irregular and of mild degree.

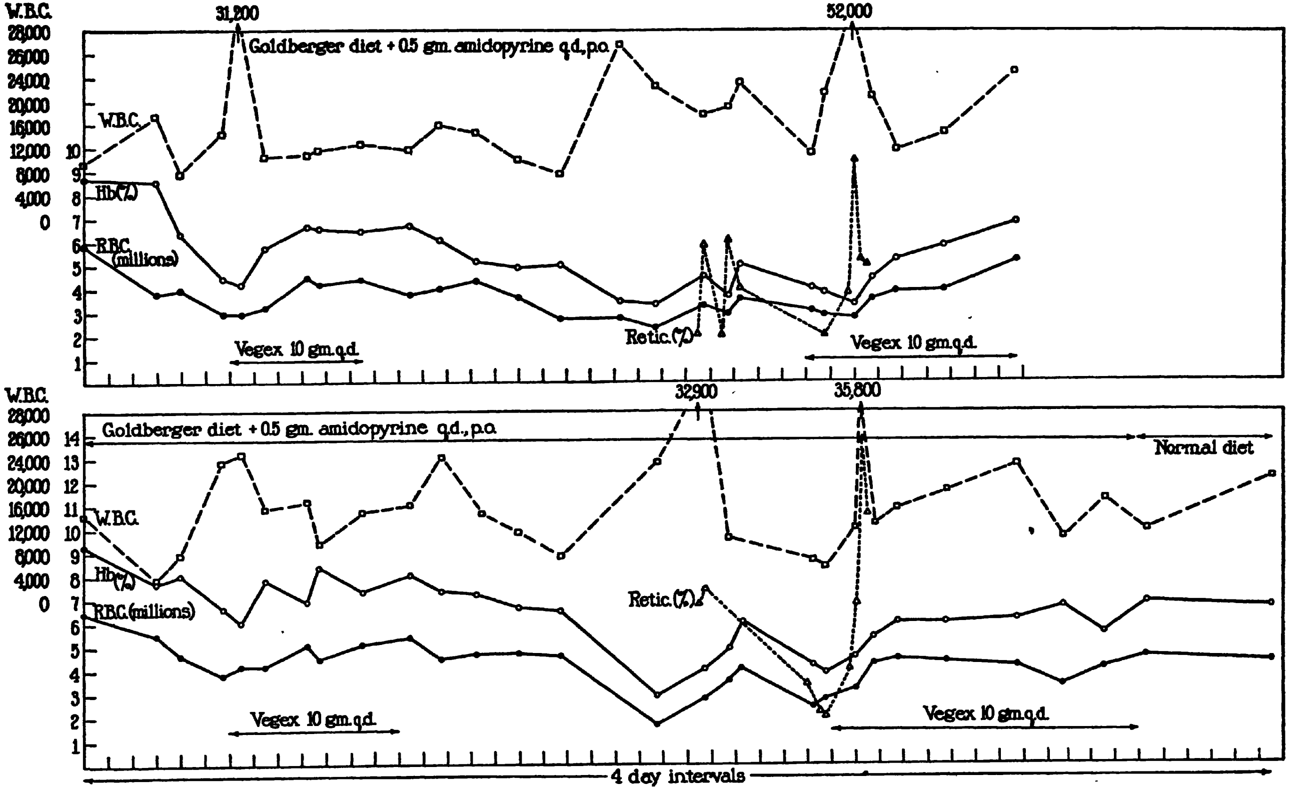
TABLE IV

High and Low Blood Levels in Dogs Receiving Normal Diet and Amidopyrine, 2 Gm. Daily

Dog No.	Before amidopyrine		Time on amido-pyrine	After amidopyrine		Remarks
	R.B.C. per c. mm.	Hb		R.B.C. per c. mm.	Hb	
	millions	*per cent*	*days*	*millions*	*per cent*	
4	6.29	89	15	3.59	69	Amidopyrine discontinued. Recovered
5	5.31	80	17	3.29	63	" "
6	5.07	75	9	2.82	43	" "
7	6.29	96	12	3.62	66	Died after convulsion
8	5.70	93	17	2.81	48	Died, pneumonia



TEXT-FIG. 4


W.B.C.
31,200
52,000
Goldberger diet + 0.5 gm. amidopyrine q.d., p.o.
28,000
26,000
24,000
20,000
16,000
12,000
8,000
4,000
0
10
9
8
7
6
5
4
3
2
1
W.B.C.
Hb(%)
R.B.C. (millions)
Retic.(%)
Vegex 10 gm. q.d.
Vegex 10 gm. q.d.
W.B.C.
32,900
35,800
Goldberger diet + 0.5 gm. amidopyrine q.d., p.o.
Normal diet
28,000
26,000
24,000
20,000
16,000
12,000
8,000
4,000
0
14
13
12
11
10
9
8
7
6
5
4
3
2
1
W.B.C.
Hb(%)
R.B.C. (millions)
Retic.(%)
Vegex 10 gm. q.d.
Vegex 10 gm. q.d.
4 day intervals

TEXT-FIG. 5

Experiment 5. Text-Fig. 5

As a supplement to the studies which have been described, this experiment tested the effect of administering 0.5 gm. of amidopyrine daily to 2 dogs over long periods of the feeding of both good and bad diets.

The results are presented in Text-fig. 5. The administration of the drug during a period of normal diet feeding was without marked effect. When the black tongue diet was fed and the drug administration was continued, there was a pronounced decline of the blood values to low levels. When vegex, a commercial yeast preparation, was administered in amounts which were known to be effective in the prevention of black tongue, a remission of the anemia was effected. The blood was maintained at levels approaching the normal as long as the vegex supplement was continued, but fell when it was omitted. This was true in spite of the fact that the amidopyrine administration was continued throughout the course of the study.

DISCUSSION

In the experiments reported, anemia was brought about in dogs fed the black tongue diet by the administration of amounts of amidopyrine which were without effect when given to dogs fed a normal diet. When sufficient amounts of the drug were administered to dogs fed the normal diet, an anemia did result. It was not as severe as that caused by administering amidopyrine to animals on the inadequate diet and was not associated with stomatitis. Ulceration of the oral and pharyngeal mucous membrane was a striking feature when amidopyrine administration was combined with the inadequate diet. That the anemia and stomatitis were not due to the diet alone is clear from the fact that they occurred before they could be expected if only the special diet was fed. Moreover, they were more severe than are the changes of black tongue and were of a somewhat different character.

The absence of leukopenia was remarkable, particularly since it would have been expected, at least in a mild degree, from the histological appearance of the bone marrow. From this fact it would appear that the action of amidopyrine is neither exactly that of benzol nor of the black tongue diet alone. On the other hand, the toxic effect on the bone marrow of the administration of an aromatic compound is so dependent upon such factors as dosage, diet, route of administration, and host susceptibility that it is difficult to prognosticate what the effect will be in a given instance.

The histological alterations of the bone marrow were of particular interest. They appeared to be similar to those described in a case of benzol poisoning by Andersen (15) and also to those seen in several cases of aplastic anemia studied in this laboratory in which no history of exposure to benzol was available.

One aspect of the experiments deserves special consideration; the fact that the combination of amidopyrine administration and the diet feeding gave a pathological result not wholly dissimilar from that following a sufficiently long period of the diet feeding alone. The stomatitis was similar to that of some cases of acute black tongue, though different in minor details from most. Anemia of mild degree has been reported to occur irregularly in recurrent, chronic black tongue by Rhoads and Miller (16) and Spies (17). A suppression of maturation of the hematopoietic elements of the bone marrow has been described in both acute and chronic black tongue by the same authors. The suggestion may be advanced that the factor in the diet which makes it productive of black tongue is an aromatic compound or that the diet renders the body incapable of detoxifying some aromatic compound, either present in the diet or a product of intrinsic metabolic formation. Further experiments dealing with this subject are in progress.

SUMMARY AND CONCLUSION

1. By feeding dogs a black tongue diet and at the same time administering amidopyrine, acute stomatitis and anemia may be produced.
2. Both stomatitis and anemia occur some time before they could be expected to appear as result of the diet feeding alone.
3. The anemia is associated with suppression of maturation of the hematopoietic bone marrow elements.

BIBLIOGRAPHY

1. Kracke, R. R., and Parker, F. P., *J. Lab. and Clin. Med.*, 1933–35, **19**, 799.
2. Madison, F. W., and Squier, T. L., *J. Am. Med. Assn.*, 1934, **102**, 755.
3. Watkins, C. H., *Proc. Staff Meetings Mayo Clinic*, 1933, **8**, 713.

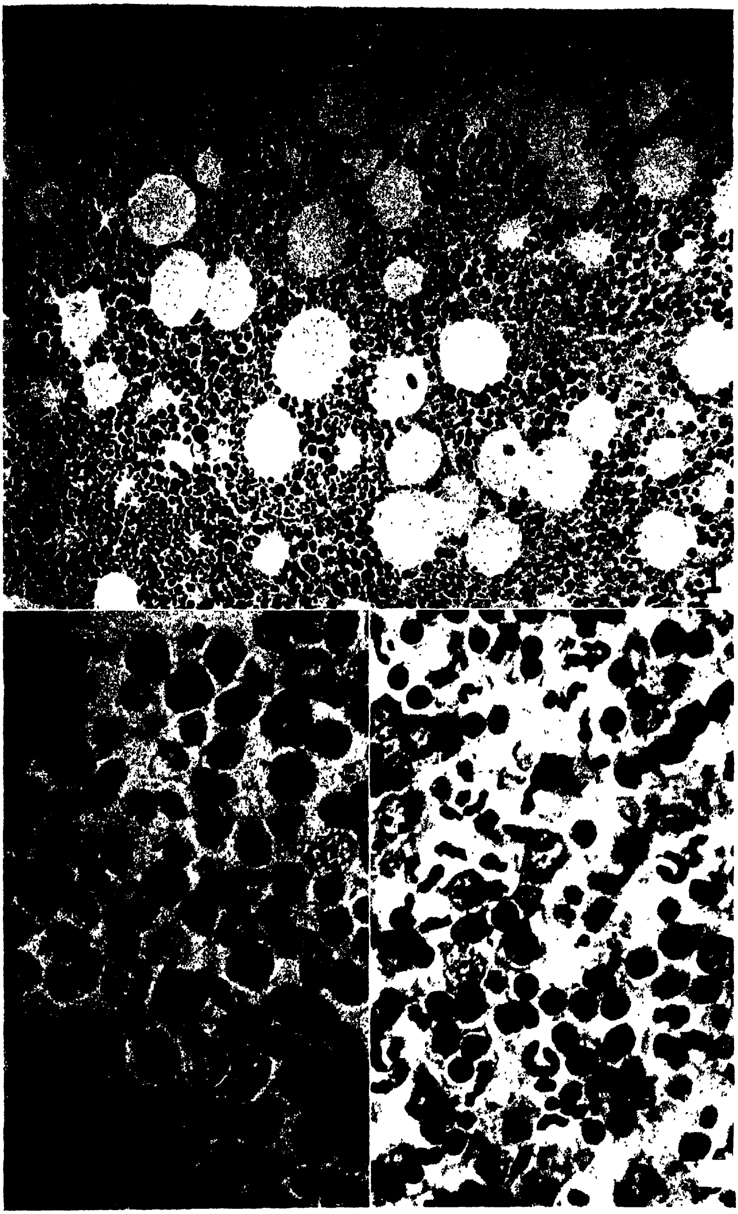
4. Sturgis, C. C., and Isaacs, R., *Tr. Assn. Am. Physn.*, 1934, **49**, 328.
5. Miller, D. K., *Science*, 1934, **80**, 320.
6. Climenko, D. R., *Proc. Soc. Exp. Biol. and Med.*, 1934–35, **32**, 823.
7. Miller, D. K., and Rhoads, C. P., *J. Exp. Med.*, 1935, **61**, 173.
8. Hamilton, A., *Arch. Path.*, 1931, **11**, 626.
9. Kracke, R. R., *J. Lab. and Clin. Med.*, 1931–32, **17**, 993.
10. Turnbull, H. M., *Proc. Roy. Soc. Med.*, 1916–17, **10**, 47.
11. Biberfeld, J., *Biochem. Z.*, Berlin, 1914, **65**, 479.
12. Goldberger, J., Wheeler, G. A., Lillie, R. D., and Rogers, L. M., *Pub. Health Rep., U. S. P. H. S.*, 1928, **43**, 1385.
13. Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Volume II. Methods, Baltimore, The Williams & Wilkins Co., 1932, 337.
14. Sabin, F. R., Miller, F. R., Smithburn, K. C., Thomas, R. M., and Hummel, L. E., *J. Exp. Med.*, 1936, **64**, 97.
15. Andersen, D. H., *Am. J. Path.*, 1934, **10**, 101.
16. Rhoads, C. P., and Miller, D. K., *J. Exp. Med.*, 1933, **58**, 585.
17. Spies, T., *Am. J. Physiol.*, 1935, **25**, 114.

EXPLANATION OF PLATE 10

Fig. 1. Photomicrograph of the bone marrow of dog 2. The marrow is less cellular than normal and the degree of cell differentiation is reduced. Mallory's eosin and methylene blue. ×250.

Fig. 2. Photomicrograph of the bone marrow of dog 2. Mallory's eosin and methylene blue. ×1000.

Fig. 3. Photomicrograph of the normal canine femoral bone marrow. Mallory's eosin and methylene blue. ×1000.



Photographed by Joseph B. Haulenbeck

(Miller and Rhoads: Damage by amidopyrine to hematopoietic function)

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CLINICAL COURSE AND TREATMENT OF SPRUE

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Job, chap. 30, verse 27: "My bowels boiled, and rested not; the days of affliction prevented me."

Job, chap. 32, verse 19: "Behold, my belly is as wine which hath no vent; it is ready to burst like new bottles."

Tropical sprue as a medical problem is confined to certain sharply delineated geographic areas. As a problem in pathologic physiology, however, the disease has been shown in recent years to be of considerable general importance and interest. The recent reviews of Castle, Rhoads, Lawson and Payne,[1] of Fairley[2] and of Reed[3] have all dealt at length with the history and symptomatology of tropical sprue. Consideration of these reports, however, reveals that a certain difference of opinion exists as to the most effective means of treating sprue and indeed as to the basic mechanism of the disease process. The patients reported on by Reed and by Fairley were treated both by dietary means and by the administration of liver extract, whereas those reported on by Castle and his associates were treated uniformly with liver extract alone, but they were followed for too short a time to allow accurate estimations of the effect of therapy.

A clinical study of thirty-three patients with sprue has been made in this hospital during the past four and a half years. Information is available concerning twenty-nine of these patients over periods varying from three months to four and a half years after they were discharged from the institution. The salient facts concerning these patients both during hospitalization and after their discharge are presented in this report.

MATERIAL

All except four of the thirty-three patients studied had resided in tropical or subtropical countries; eleven had lived in China, nine in

Puerto Rico, four in Central or South America and one each in Cuba, Turkey, Algeria, Japan and the Philippine Islands. Twenty-five of the patients were females, and eight were males.

In this report the patients have been divided into three groups according to the presence or absence of certain symptoms and clinical findings at the time of the patients' admission to this hospital. All the patients gave histories which were characteristic of sprue, but in this report the histories are disregarded as far as the grouping is concerned. Group A includes those patients who presented the classic picture of severe sprue when first admitted to the hospital; all of them had diarrhea, gastro-intestinal distress and well marked anemia. Group B is made up of those patients who suffered from diarrhea and gastro-intestinal distress but showed no significant degree of anemia. Group C comprises those who presented neither diarrhea nor anemia when they came under observation but complained only of abdominal discomfort and distention.

Group A: Patients with Anemia and Diarrhea on Entry.—Ten patients presenting the classic picture of severe tropical sprue make up this group. Eight had resided in tropical or subtropical countries, but the other two had never been south of Florida or Texas, respectively. The clinical pictures presented by the last two patients differed in no respect from those seen in the others of the group.

Gastro-Intestinal Symptoms (Table 1): Recurrent disturbances of the gastro-intestinal tract had been present for periods varying from four to twenty years. The most prominent and the most distressing of these symptoms was diarrhea, which had occurred in all the patients. Vomiting had been a prominent feature at one time or another in six of the patients, while nausea was a complaint common to the entire group. Abdominal distention and discomfort had occurred in all.

At the time of the patient's admission to the hospital the stools varied in number from four to twenty per day and were in general voluminous and yellow, with a yeasty odor. In certain instances, however, they were watery and not to be distinguished from any other diarrheal stool. Besides the diarrhea these patients complained of abdominal discomfort associated with cramps, flatulence, distention, borborygmi and the eructation of gas.

Loss of Weight (Table 2): Nine of the patients had lost weight over periods of from one to sixteen years. The amount of weight lost varied from 13.6 to 53.6 Kg., and all nine patients were markedly emaciated when admitted. After treatment six patients gained weight rapidly, the weight of one patient being increased by 15 Kg. in less than six weeks.

Oral Symptoms (Table 3): Recurrent soreness of the tongue and mouth had been noticed by nine of these patients over periods varying from one to fifteen years. In most instances the onset of the oral symptoms took place some time after the onset of the gastro-intestinal symptoms. Two types of soreness of the mouth were reported. One type consisted of redness and burning of the tongue and the other of ulceration of the buccal and gingival mucous membrane, accompanied at times with pain and burning of the whole oral cavity. Salivation was frequently a distressing symptom.

At the time of admission to the hospital nine of the patients had visible oral lesions, which consisted of (1) atrophy of the lingual papillae, (2) redness of the tongue and oral mucosa and (3) vesicular and ulcerated lesions of the tongue and mucous membrane. These various types of oral lesions occurred singly or in any combination. Six patients in this group had atrophic glossitis; one of these had aphthous lesions of the tongue, and another had similar lesions of the oral mucosa. Redness of the tongue was present in three patients; one of these showed also reddening of the oral mucosa, and two of them had aphthae as well.

Gastric Analysis (Table 4): A standard method of gastric analysis was employed, consisting of the withdrawal of four specimens of gastric juice. The first specimen was taken from the stomach during fasting, the second twenty minutes after an alcohol test meal, the third forty minutes after the alcohol meal and the fourth twenty minutes after the intramuscular injection of 0.5 mg. of histamine. In table 4 the volume of gastric juice obtained and the amount of free hydrochloric acid present in each specimen are tabulated.

Failure of the stomach to secrete free hydrochloric acid after the injection of histamine was observed in only one patient (case 6). In four other patients free hydrochloric acid was absent until after the

TABLE 1

Gastro-Intestinal Symptoms

Case	Patient	Sex	Before Entry into Hospital				At Entry	
			Vomiting	Flatulence	Diarrhea	Years Before Entry	Flatulence	Diarrhea
				Group A				
1	R. D.	F	+	+	+	7	+	+
2	M. F.	F	+	+	+	4	+	+
3	M. G.	F	+	+	+	18	+	+
4	B. M.	F	+	+	+	7	+	+
5	L. N.	F	+	+	+	20	+	+
6	G. M.	M	0	+	+	16	+	+
7	E. M.	F	+	+	+	9	+	+
8	C. O.	F	0	+	+	20	+	+
9	L. S.	F	0	+	+	15	+	+
10	R. S.	F	0	+	+	7	+	+
				Group B				
11	M. B.	F	+	+	+	4	+	+
12	R. B.	M	+	+	+	9	+	+
13	A. D.	F	0	+	+	2½	+	+
14	F. E.	F	0	+	+	1	+	+
15	M. L. F.	F	0	+	+	11	+	+
16	P. M.	M	0	+	+	8	+	+
17	M. P.	M	0	+	+	1½	+	+
18	E. R.	F	0	+	+	6	+	+
19	H. W.	M	0	+	+	1	+	+
				Group C				
20	R. C.	M	0	+	+	1	+	0
21	A. DeG.	F	0	+	+	3	+	0
22	R. F.	F	0	+	+	15	+	0
23	F. H.	F	+	+	+	24	+	0
24	L. H.	F	0	+	+	2	+	0
25	P. K.	M	0	+	+	13	+	0
26	G. L.	M	0	+	+	3	0	0
27	E. P.	F	+	+	+	5	0	0
28	R. P.	F	0	+	+	7	0	0
29	M. P.	F	+	+	+	12	+	0
30	E. P.	F	+	+	+	6	+	0
31	B. R.	F	+	+	+	25	+	0
32	E. S.	F	0	+	+	8	+	0
33	A. W.	F	0	+	+	1	+	0

TABLE 2

Loss of Weight

Case	Loss of Weight, Kg.	Duration of Symptoms	Weight at Entry, Kg.	Weight at Discharge, Kg.
Group A				
1	+	7 years	41.5	54.9
2	53.6	4 years	42.0	57.0
3	13.6	5 years	45.2	42.0
4	+	7 years	55.9	52.4
5	43.2	15 years	52.5	55.7
6	+	16 years	45.9	48.6
7	22.4	1 year	31.4	42.4
8	0	....	68.7	68.0
9	21.8	3 years	35.4	37.3
10	17.3	7 years	43.7	43.1
Group B				
11	9.1	4 years	44.7	45.7
12*	+	9 years	63.8	63.6
			69.8	71.4
13	18.2	2½ years	58.3	56.8
14	+	11 months	48.4	49.0
15	0	....	55.0	56.7
16	0	....	70.6	70.2
17	+	14 months	50.1	49.9
18	0	....	65.7	67.4
19	+	1 month	56.8	57.6
Group C				
20	0	....	76.2	....
21	+	3 years	51.9	53.0
22	0	....	60.4	61.5
23	4.6	2 years	56.4	56.7
24	12.3	2 years	49.0	50.1
25	13.6	3 years	59.7	59.8
26	13.6	3 years	93.2	89.2
27	0	....	73.2	....
28	+	14 months	50.1	49.9
29	0	....	61.5	62.4
30	+	6 years	....	....
31	9.1	10 years	41.9	42.7
32	0	....	52.2	....
33	18.2	2 years	....	....

* The patient was admitted to the hospital on two different occasions.

injection of histamine. In only one patient (case 10) was the amount of free hydrochloric acid greater than 30, and in most instances the volume of gastric juice obtained was small.

Blood Picture (Table 5): Nine of the patients gave a history of having had anemia before admission to the hospital. In one instance the anemia had been present for ten years before entry, but in the other cases it was of more recent origin. The exact nature of the type of anemia which was present before admission was unknown except for one patient (case 7), in whom microcytic hypochromic anemia had been noted. A red blood cell count of less than 3,500,000 per cubic millimeter was arbitrarily selected as indicative of distinct anemia, since the frequent macrocytosis warranted the employment of erythrocyte counts rather than hemoglobin values as a criterion.

At the time of admission to the hospital all of these ten patients had red blood cell counts of less than 3,500,000, and the erythrocyte levels of two were below 1,000,000. The hemoglobin values were relatively high, giving all the patients, except in cases 6 and 9, a definitely macrocytic hyperchromic blood picture. The color index and the mean corpuscular volume were well above the normal range and in many instances were at levels as high as those seen in pernicious anemia. In cases 6 and 9 the anemia was of a normocytic type.

As in pernicious anemia, the number of white blood cells was decreased, in six cases below 5,000. In only one was the count more than 6,000.

By comparing the red blood cell count at the time of the patient's discharge with that on entry, the effect of antisprue therapy on the hematopoietic system is evident. More detailed studies of the blood are shown in the two accompanying charts (figs. 1 and 2; cases 4 and 5, respectively). In each of these patients a rise in the reticulocyte count occurred and was followed by a remission.

The two following abstracts of cases are given to illustrate the type of patient included in this group:

CASE 4.—B. M., a 37 year old American woman, entered the hospital complaining of attacks of diarrhea for seven years. She had resided in Cuba for twelve years. Seven years before entering the hospital she began to have attacks of diarrhea accompanied with nausea, vomiting and abdominal cramps. Two years after the onset of these attacks the patient moved to a temperate climate and was

TABLE 3

Oral Symptoms

Case	Sore Tongue	Sore Mucous Membrane	Period of Observation Before Entry	Observed at Entry
Group A				
1	+	+	7 years	Atrophy of papillae
2	+	+	4 years	Atrophy of papillae
3	+	+	5 years	Atrophy of papillae
4	+	+	5 years	Atrophy of papillae; aphthae of mucous membrane
5	+	..	15 years	Aphthae of tongue; atrophy of papillae
6	+	+	2 years	Red, smooth tongue; aphthae, red mucosa
7	+	+	1 year	Red, smooth tongue
8	+	+	5 years	Red tongue; aphthae of mucosa
9	0	0	....	
10	+	+	7 years	Atrophy of papillae
Group B				
11	+	+	3½ years	Aphthae of mucosa
12	+	+	9 years	
13	+	+	1½ years	Atrophy of papillae; aphthae of mucous membranes
14	+	+	11 months	Red tongue
15	+	+	8 years	Aphthae of mucous membrane
16	+	+	1½ years	
17	+	+	4 months	Red, smooth tongue; aphthae of tongue and mucosa
18	+	+	6 years	Atrophy of papillae; aphthae of mucous membrane
19	+	+	6 months	Red tongue; atrophy of papillae
Group C				
20	0	0	....	
21	+	+	3 years	
22	0	+	10 years	Tongue red along edges
23	+	+	2 years	Tongue red along edges; atrophy of papillae
24	0	+	1 year	
25	+	0	13 years	Slight atrophy of papillae
26	+	+	4 years	Slight atrophy of papillae; tongue red at tip
27	0	+	1 year	Slight atrophy of papillae
28	+	+	7 years	
29	+	+	6 months	Aphthae of mucosa; tongue smooth
30	+	+	6 years	
31	0	+	3 years	
32	+	+	3 years	Redness and aphthae of tongue and mucous membrane
33	+	0	....	

TABLE 4

*Gastric Analyses**

Case	Volume, Cc.				Free Hydrochloric Acid			
	I	II	III	IV	I	II	III	IV
				Group A				
1	17	15	15	28	0	0	0	17
3	30	20	15	25	0	0	20	79
4	35	20	12	19	0	4	10	13
5	25	21	12	16	0	0	0	7
6	20	30	15	30	0	0	0	0
7	16	42	8	24	0	0	0	25
8	38	62	18	....	0	0	24	11
9	40	25	15	30	0	0	0	36
10	86	35	30	....	6	62	122	....
Group average	33.0	30.0	15.5	24.6	0.7	7.3	19.5	23.5
				Group B				
12	....	....	....	....	20	36	....	96
13	12	11	9	....	0	6	8	....
14	26	15	25	....	0	9	31	....
15	12	20	24	13	0	0	0	0
16	....	....	....	....	11	....	....	22
18	15	....	22	13	0	....	10	16
19	30	35	30	20	14	67	91	99
Group average	19.0	20.3	22.0	15.3	6.4	23.6	28.0	46. 6
				Group C				
20	23	15	10	20	0	0	0	0
21	30	25	20	35	0	23	37	70
22	20	10	15	30	5	9	20	38
23	22	20	15	....	0	0	12	....
24	10	12	15	30	0	8	14	31
25	30	75	25	40	0	30	50	97
26	....	....	....	....	0	3	58	....
27	14	15	10	25	0	0	14	32
28	66	42	30	....	25	25	56	....
29	40	9	15	....	6	27	30	....
30	55	40	15	60	0	24	20	53
31	30	10	12	16	2	8	14	20
32	30	12	32	40	0	0	0	0
33	10	10	24	40	10	18	24	40
Group average	29.2	22.5	18.3	33.6	3.4	12.5	24.9	38.1
Series average	29.0	24.8	18.0	27.7	3.3	12.8	23.8	30.5

* I represents data for specimen taken during fasting: II, specimen taken twenty minutes after an alcohol test meal; III, specimen taken forty minutes after test meal; IV, specimen taken twenty minutes after injection of histamine. The volumes under specimen I do not always represent actual volumes during fasting, as some of the patients were allowed to sip water while swallowing the tube.

TABLE 5

Data on the Blood

Case	Anemia Observed Before Entry	Values at Entry					Values at Discharge				
		Erythrocytes, Millions per Cu.Mm.	Hemoglobin, Percentage	White Blood Cells Per Cu.Mm.	Mean Corpuscular Volume, Cu. Microns	Color Index	Erythrocytes, Millions per Cu.Mm.	Hemoglobin, Percentage	White Blood Cells, Per Cu.Mm.	Mean Corpuscular Volume, Cu. Microns	Color Index
						Group A					
1	1 yr.	0.82	22	2,100	131	1.34	4.17	76	5,200	92	0.92
2	1 yr.	1.13	30	3,250	115	1.33	3.45	78	6,750	102	1.12
3	+	0.90	30	1,400	121	1.67	3.67	77	3,800	101	1.05
4	5 yr.	2.05	67	2,050	135	1.63	3.98	92	7,250	100	1.16
5	1½ yr.	1.38	35	1,150	102	1.27	3.04	67	6,650	97	1.12
6	+	3.10	59	9,600	91	0.95	3.90	80	6,700	101	1.03
7	10 yr.	3.30	70	5,100	...	1.06	4.90	94	.....	...	0.96
8	5 yr.	2.96	79	5,400	138	1.34	3.85	82	7,700	100	1.06
9	3 mo.	3.15	53	4,650	78	0.84	3.47	62	5,300	79	0.90
10	0	3.12	81	5,500	118	1.30	....	..	.....	...	....
Group average		2.19	53	4,020	114	1.27	3.83	79	6,170	96.5	1.04
						Group B					
11	4 yr.	4.61	97	12,850	90	1.05	4.97	100	12,250	86	1.05
12	5 yr.	3.59	92	5,150	113	1.28	4.68	94	7,950	90	1.01
13	2 yr.	3.67	87	7,700	108	1.19	....	..	.....	...	....
14	11 mo.	4.69	77	6,100	74	0.83	5.36	77	8,900	70	0.72
15	0	4.40	95	.....	111	1.08	....	..	.....	...	....
16	6 mo.	4.64	105	5,900	95	1.14	4.37	105	6,350	102	1.20
17	2 mo.	3.52	58	7,150	...	0.83	3.87	59	9,900	75	0.77
18	5 yr.	4.02	83	7,000	94	1.04	....	..	.....	...	....
19	0	4.47	86	8,300	91	0.98	4.54	94	.....	102	1.04
Group average		4.18	87	7,520	97	1.05					
						Group C					
20	0	4.42	99	4,100	...	1.12	....	..	.....	...	....
21	8 mo.	3.57	97	4,150	...	1.36	4.06	94	5,250	108	1.17
22	10 yr.	5.05	99	4,000	84	0.98	4.62	90	4,300	86	0.98
23	+	3.48	90	7,550	103	1.30	3.39	92	5,400	115	1.36
24	0	4.08	91	6,500	99	1.12	4.13	84	7,950	92	1.12
25	0	3.87	96	4,150	106	1.24	....	..	.....	...	....
26	3 mo.	4.18	106	4,300	110	1.27	....	..	.....	...	....
27	0	3.94	100	2,450	72	1.27	....	..	.....	...	....
28	0	3.94	90	9,350	...	1.14	....	..	.....	...	....
29	0	4.57	96	7,400	89	1.05	....	..	.....	...	....
30	6 yr.	3.72	89	6,350	101	1.20	....	..	.....	...	....
31	0	3.96	83	7,600	82	1.05	....	..	.....	...	....
32	3 yr.	3.60	91	8,600	115	1.27	3.85	96	7,000	104	1.25
33	0	3.51	90	10,150	102	1.28	3.57	83	8,600	...	....
Group average		3.99	94	6,190	97	1.19					

free from symptoms for six months. At the end of that time the diarrhea returned, with soreness of the tongue and mouth. Anemia of severe degree was present. Liver extract was given by mouth; the diarrhea ceased, and the red blood cell count rose to 4,000,000 per cubic millimeter. The patient was well for one year; then the diarrhea and soreness of the mouth recurred but were alleviated by the use of a diet of meat and eggs. The anemia was not relieved by this diet, and two transfusions were given. She was well until one year before admission to the hospital, when the diarrhea recurred and the high protein diet afforded no relief. About two months before she entered the hospital weakness and pallor appeared and were not relieved by orally administered liver extract.

Physical Examination.—The patient was fairly well nourished but was pale. The tip and dorsum of the tongue showed atrophy of the papillae. There was a small yellow ulcer on the mucosa of the upper lip. The abdomen was distended and tympanitic.

Laboratory Examination.—Gastric analysis showed free hydrochloric acid in all specimens except the one taken during fasting. There were 2,000,000 erythrocytes per cubic millimeter, 66 per cent hemoglobin, a color index of 1.65 and a mean corpuscular volume of 135 cubic microns.

Course.—During the first three days the patient had from ten to fifteen bowel movements a day. She was then given daily intramuscular injections of 10 cc. of liver extract,[4] an amount derived from 50 Gm. of whole liver. On the tenth day the reticulocyte count reached a peak of 12.8 per cent (fig. 1). This was followed by an improvement in the blood values, and the patient was discharged from the hospital two months after entry, with an erythrocyte count of 3,980,000 and a hemoglobin value of 92 per cent. After discharge she received 10 cc. of liver extract every two weeks by intramuscular injection, and on this regimen she was symptom free for nearly a year.

Nine months after discharge the patient went to Florida and discontinued the injections of liver extract. Two months later diarrhea recurred, and the patient lost her appetite and grew progressively weaker. From that time until her return to the hospital, five months later, attacks of diarrhea, soreness of the mouth and tongue, loss of weight and weakness became progressively worse. When readmitted she was having from fifteen to twenty evacuations daily.

Physical Examination.—The patient was emaciated. The tongue was red at the tip and along the margins. The buccal mucosa was injected, and there were several aphthous lesions. The abdomen was moderately distended, and peristaltic rushes were audible.

Laboratory Examinations.—The results showed: erythrocytes, 2,230,000; hemoglobin, 62 per cent; color index, 1.39, and mean corpuscular volume, 115 cubic microns.

Course.—The patient was given 10 cc. of liver extract by intramuscular injection daily for five days. Soreness of the tongue disappeared after the first injection, and the aphthous ulcers healed rapidly. Diarrhea ceased after the third injection. The number of injections of liver extract was decreased to two a week

In ten days the patient gained 2 Kg. in weight. One month later the erythrocyte count was 3,350,000, and the hemoglobin value was 85 per cent. Injections of 10 cc. of liver extract intramuscularly have been continued twice each week, and with this therapy the patient has remained essentially symptom free.

CASE 5.—L. N., an American woman aged 45 who had resided in China for twenty-one years, had suffered from sprue for fifteen years. The presenting symptoms were stomatitis, glossitis, proctitis and diarrhea. Nine years before her entry into the hospital a remission had followed the use of a diet with a high protein, a low fat and a low carbohydrate content. For three years before her admission to the hospital nausea, vomiting and diarrhea, with marked loss of weight, had been present. For six months the patient had been in bed, able to retain only milk and toast. Twice a week during the three months immediately preceding her admission to the hospital she had received an intramuscular injection of 2 cc. of liver extract, an amount derived from 10 Gm. of whole liver.

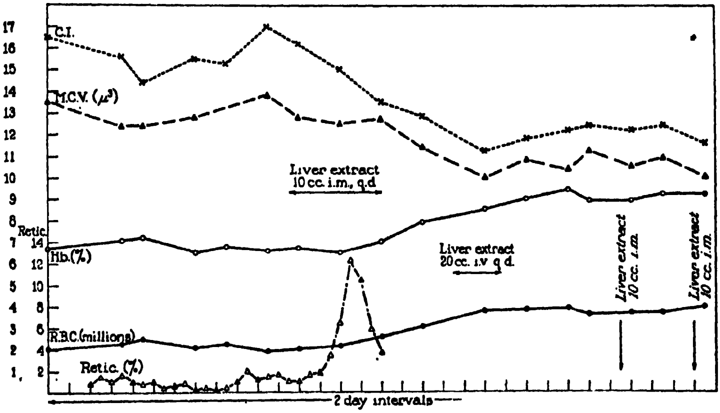

FIG. 1 (case 4).—Chart of the changes in the blood following the parenteral administration of liver extract. In figures 1 and 2 the values for the color index (*C. I.*) have been multiplied by 10; the values for the mean corpuscular volume (*M. C. V.*) in cubic microns have been divided by 10; the percentage values for hemoglobin (*Hb.*) have been divided by 10, and the values for erythrocytes (*R. B. C.*) have been divided by 1,000,000.

Physical Examination.—The patient was markedly emaciated. Pallor and faint yellowness of the skin and mucous membranes were noted. The tongue showed atrophy of the papillae. There was edema of the legs.

Laboratory Examination.—The results showed: erythrocytes, 1,380,000; hemoglobin, 35 per cent; white blood cells, 1,150; mean corpuscular volume, 102 cubic microns; color index, 1.27. Gastric analysis showed free hydrochloric acid only after the injection of histamine.

Course.—The patient was given 20 cc. of liver extract[5] intravenously each day during her stay in the hospital. On the seventh day of treatment the reticulocyte value rose to 43.2 per cent. This reticulocyte response was followed by an increase in the erythrocyte count and in the hemoglobin value (fig. 2). Diarrhea ceased soon after the injections of liver were begun. The patient's appetite improved, and she was discharged three weeks after entry, with an erythrocyte count of 3,040,000 and a hemoglobin value of 67 per cent.

After discharge from the hospital the patient moved to Alabama. She has received an intramuscular injection of 10 cc. of liver extract every two weeks, and with this therapy she has remained free from symptoms for the past two and a half years.

Summary: These ten patients with severe sprue have been grouped together because of the fact that anemia as well as gastro-intestinal disturbance was present at the time of admission to the hospital. The histories of two patients in this group are presented. Case 4 demonstrates the therapeutic failure of orally administered liver extract, as well as the necessity of continued therapy. Case 5 is presented because it is typical of severe sprue and illustrates the dramatic results that can be obtained by intensive therapy with parenterally administered liver extract without change of diet.

Group B: Patients With Diarrhea but Without Anemia.—This group is made up of nine patients who had distention and diarrhea but no anemia at the time of admission to the hospital. The lack of disturbance of hematopoiesis separates these patients from those just described. Seven members of this group had resided in tropical or subtropical countries, whereas two had not been out of the temperate zone.

Gastro-Intestinal Symptoms (Table 1): Recurrent attacks of distention and diarrhea had occurred in every instance over periods varying from one to nine years. The gastro-intestinal symptoms did not differ from those of the patients in group A, except that nausea and vomiting had been less frequent and the attacks of diarrhea had not extended over so long a period.

At the time of admission to the hospital all these patients had diarrhea. The stools were on the whole not so numerous as those of the patients of group A but were of similar type.

Loss of Weight (Table 2): Six of the nine patients had lost weight over periods of from one month to nine years, but the average loss was not as great as in the preceding group, and the patients were not so emaciated. Since the period of hospitalization was relatively short, the gain in weight was correspondingly less striking in this group.

Oral Symptoms (Table 3): The oral symptoms of these patients were identical with those of the patients in group A. All of these patients had noticed soreness of the tongue or of the mouth over periods varying from four months to nine years. In most instances the oral symptoms first developed after the onset of the diarrheal attacks.

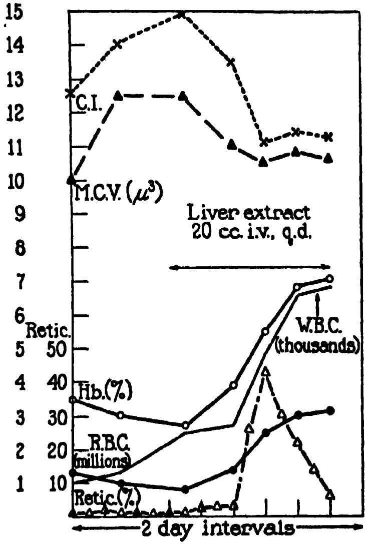

FIG. 2. (case 5).—Chart of the changes in the blood following the parenteral administration of liver extract.[5]

The same types of oral lesions were observed as have been described for the patients with more severe involvement. Two of these patients had no oral changes when they were admitted to the hospital, although they both gave a history of soreness of the mouth. Four patients had aphthous lesions of the oral mucosa, and two of these showed also atrophic glossitis. Three other patients were suffering from acute glossitis, with redness of the tongue; of these, one had atrophy of the papillae as well, and another had aphthous ulcers of the buccal mucosa.

Gastric Analysis (Table 4): Gastric analyses were carried out for seven patients of this group. In only one patient (case 15) was no free hydrochloric acid found in the gastric juice after the injection of histamine. The other six patients showed free hydrochloric acid either in the specimen taken during fasting or after the alcohol test meal. The quantities of gastric juice obtained were small.

Blood Picture (Table 5): Some of the nine patients gave a history of having suffered from anemia at some time during the course of their illness. However, at the time of admission to the hospital all the patients had red blood cell counts above 3,500,000. One patient (case 17) showed a hemoglobin value of 58 per cent; nevertheless, he is included in this group, since the erythrocytes numbered 3,520,000. Three of the patients had erythrocyte counts between 3,500,000 and 4,000,000; three had counts between 4,000,000 and 4,500,000, and three had counts between 4,500,000 and 5,000,000. In three instances the mean corpuscular volume and the color index were above the normal range, giving these three patients (cases 12, 13 and 15) a slightly macrocytic hyperchromic blood picture. In all the remaining cases the blood was normocytic.

CASE 12.—R. B., a white man aged 56, had suffered from sprue for nine years before admission to the hospital. He had resided in Puerto Rico for five years when diarrhea first developed. This attack was characterized by large foamy stools and was accompanied with nausea and vomiting. Soon after the onset of the gastro-intestinal symptoms his tongue became sore, and salivation increased. During the next two years diarrhea, consisting of two or three spruelike stools, persisted, and the tongue was sore from time to time. Four years after the onset of symptoms the patient returned to the United States. At that time he had lost about 15 pounds (7 Kg.) in weight; diarrhea and soreness of the tongue and mouth were still present. The administration of liver by mouth was followed by striking improvement. When he discontinued liver therapy a year later, diarrhea again developed, and he began to lose weight. For fifteen months he was given daily intramuscular injections of 3 cc. of liver extract.[4] On this regimen he felt well but finally tired of the injections and discontinued them. He was then given three vials of liver extract[4] a day by mouth with the result that diarrhea and the loss of weight soon recurred, along with increasing fatigue. He was given 2 cc. of liver extract by intravenous injection once a week, with no improvement in his symptoms. There was no response to a strict dietary regimen for sprue.

Physical Examination.—The patient was well developed and fairly well nourished but appeared to be chronically ill. There were raised, rough, granular patches of skin over the elbows and knees. The tongue and mouth showed no abnormality.

Laboratory Examinations.—Gastric analysis showed free hydrochloric acid in all specimens. Study of the blood showed: erythrocytes, 3,590,000; hemoglobin, 92 per cent; leukocytes, 5,150; color index, 1.28, and mean corpuscular volume, 113 cubic microns.

Course in Hospital.—The patient was given a diet for sprue and daily intravenous injections of 20 cc. of liver extract (the amount derived from 50 Gm. of whole liver). Dramatic relief from symptoms and complete amelioration of the gastro-intestinal disturbances resulted. The patient was discharged three and a half weeks after admission to the hospital, with instructions to take 20 cc. of liver extract by intravenous injection once a week.

Subsequent Course.—After leaving the hospital the patient received 20 cc. of liver extract intravenously every ten days. After each injection the stools were formed for three days, semiformed for three days and loose and foamy for the last four days of the interval between injections. Marked flatulence accompanied the diarrhea, but no recurrence of the stomatitis or anemia developed. In spite of the gastro-intestinal disturbances the patient gained 6.2 Kg. in the six months following his discharge from the hospital. He was then readmitted and treated with daily intravenous injections of 20 cc. of liver extract for eleven days. On the third day of this course of therapy the stools became formed and remained so during the patient's stay in the hospital. He was again discharged as symptom free and received two intravenous injections of liver extract a week after that. On this regimen he has remained symptomatically well for nine months.

CASE 15.—M. L. F., a 42 year old American woman who had resided for twelve years in southern China, had suffered from sprue for eleven years. The first attack of diarrhea occurred eleven years before her entry into the hospital and was not accompanied with fever, nausea or vomiting. Attacks of diarrhea recurred frequently during the next three years and were accompanied with soreness of the tongue and mouth. For the next five years the patient was symptom free. Three years before her entry into the hospital diarrhea with soreness of the tongue and mouth returned. After one year she was given a diet for sprue, consisting of liver, meat, fresh vegetables and fruits; on this diet she was symptom free for six months, after which her symptoms returned. For one year before her entry into the hospital the diarrhea was so severe that she was forced to give up her work and return to this country.

Physical Examination.—The patient was fairly well nourished. The tongue showed no atrophy of the papillae, but there were numerous small yellow ulcerations of the buccal mucosa. The abdomen was moderately distended and tympanitic. Peristalsis was audible.

Laboratory Examination.—Gastric analysis showed no free hydrochloric acid, even after the injection of histamine. Study of the blood showed: red blood cells, 4,400,000; hemoglobin, 95 per cent; white blood cells, 5,600; mean corpuscular volume, 111 cubic microns, and color index, 1.08.

Course.—The patient was given a diet with a high protein, a low fat and a low carbohydrate content. Five cubic centimeters of liver extract was given intramuscularly daily for five days and then 20 cc. by intravenous injection daily for eight days. With this therapy the diarrhea and flatulence ceased, and the oral lesions disappeared. The patient was discharged six weeks after entry.

After discharge the patient received intramuscular injections of 10 cc. of liver extract twice a week for one month. The interval between injections was then lengthened, depending on how long the patient remained symptom free. Nine months after discharge from the hospital the patient returned to China. Since then she has received 10 cc. of liver extract by intramuscular injection every two or three weeks and has remained entirely free from symptoms.

Summary: This group consists of nine patients who had sprue with diarrhea but without anemia. Their complaints at the time of entry were chiefly of gastro-intestinal disorders. Two typical cases of this group are presented, both of which illustrate the amelioration of intestinal dysfunction which may be obtained by the parenteral injection of liver extract, although dietary therapy has been ineffective.

Group C: Patients Without Anemia or Diarrhea.—This group includes fourteen patients who had no diarrhea or anemia at the time of admission to the hospital. All had resided in tropical or subtropical countries, and all presented symptoms which warranted the diagnosis of sprue.

Gastro-Intestinal Symptoms (Table 1): Before entry into the hospital all these patients had had repeated attacks of flatulence and diarrhea over periods varying from one to twenty-five years. These attacks did not differ from those of the patients in the preceding groups. Five patients complained of nausea and vomiting as a frequent symptom. All the patients in the group had had voluminous, frothy, yellow, foul stools at one time. The ingestion of fatty or starchy foods had aggravated their symptoms.

On admission to the hospital these patients had no diarrhea. All except three of them had severe abdominal discomfort marked by abdominal cramps, audible peristalsis, rumbling of gas and frequent expulsion of gas both by mouth and by rectum. The stools were often soft or semiformed, but many had formed stools which were of normal appearance.

Loss of Weight (Table 2): Loss of weight had taken place in ten patients over periods varying from fourteen months to ten years. For the patients who had kept accurate figures for the amount of weight lost the values ranged from 4.6 to 18.2 Kg. At entry the patients were not so emaciated as those of group A or group B.

Oral Symptoms (Table 3): The oral symptoms of these patients were similar to those of the patients in the preceding groups. Before admission thirteen of the patients had suffered from soreness of the mouth at irregular intervals over periods varying from six months to thirteen years. In seven cases soreness of both the tongue and the oral mucosa had occurred, while two patients complained of soreness of the tongue alone and four patients of soreness limited to the oral mucous membranes.

At the time of admission to the hospital seven patients presented oral lesions. Five had atrophic glossitis; of these, two showed reddening of the tongue, and one had aphthous ulcers on the buccal mucosa. Two other patients had no papillary atrophy but did have redness of the tongue; one of them had aphthous lesions of both the tongue and the oral mucosa.

Gastric Analysis (Table 4): Gastric analyses were made for all the patients of this group. In two patients (cases 20 and 32) no free hydrochloric acid was found in the gastric juice even after the injection of histamine. In two patients (cases 23 and 27) free hydrochloric acid first appeared in the specimen taken forty minutes after the administration of the alcohol test meal. In most instances the volume of gastric juice and the content of free hydrochloric acid were approximately normal. The results of the gastric analyses for these patients approached the normal more closely than did those for the patients in the other two groups.

Blood Picture (Table 5): Anemia had occurred in six of these patients at periods varying from three months to ten years before entry, but at the time of admission to the hospital it was present in none. The red blood cell counts of eight patients were between 3,500,000 and 4,000,000; the other six had counts of over 4,000,000. The hemoglobin levels for seven of the patients were relatively high in proportion to the erythrocyte counts, and the values for the mean corpuscular volume and the color index were above the normal range. Thus, these seven patients showed a moderately macrocytic hyperchromic blood picture, even though they did not have anemia. In three cases the mean corpuscular volume was not determined, but the color index was high. The remaining four patients in the group had a normocytic type of blood picture.

CASE 29.—M. P., a 57 year old American woman, was admitted to the hospital with complaints of soreness of the mouth and abdominal discomfort. For thirty-two years prior to entry she had resided in Japan. She suffered her first attack of diarrhea, accompanied with nausea and vomiting, twelve years before admission to the hospital. She recovered from this episode and remained well until six years later, when abdominal discomfort, flatulence, diarrhea and soreness of the mouth developed. These symptoms persisted irregularly until one year before her entry into the hospital, when she had a severe attack of diarrhea. This symptom then disappeared, but the soreness of the mouth and flatulence persisted.

Physical Examination.—The patient was well developed and well nourished. Two small yellow punched-out ulcers were present on the mucous membrane of the lower lip. The tongue showed atrophy of the papillae well up over the dorsum. The remainder of the examination revealed essentially normal results.

Laboratory Examinations.—Gastric analysis revealed free hydrochloric acid in all specimens. Examination of the blood gave the following results: erythrocytes, 4,570,000; hemoglobin, 96 per cent; color index, 1.05; mean corpuscular volume, 89 cubic microns, and leukocytes, 7,400.

Course.—The patient had no diarrhea on entry but had marked flatulence and abdominal discomfort. She was given a diet for sprue and intramuscular injections of 10 cc. of liver extract daily for eight days. After this treatment the symptoms were markedly abated. The ulceration of the mouth disappeared, and the tongue was no longer sore. Abdominal discomfort, though still present, was much less marked at the time of the patient's discharge from the hospital. Thereafter she received two injections of liver extract a week for one month, then one injection a week for four weeks and finally one injection every two weeks. With this course of therapy she has become practically symptom free.

CASE 32.—E. S., an American woman of 37 who had resided in Egypt and northern Africa for seven years, suffered her first attack of diarrhea six years before entry into the hospital. Five or six afebrile attacks characterized by from eight to ten loose to watery stools a day occurred during the first year of her illness. Two years later soreness of the tongue and mouth developed, diarrhea recurred frequently but was not so severe and the stools showed spruelike characteristics. Three years before entry liver extract was given by mouth, with improvement. One year later the patient was found to have microcytic anemia and was again given liver extract as well as iron by mouth. After this therapy the diarrhea ceased and had not recurred when the patient was seen in this clinic. At that time she complained chiefly of abdominal discomfort and flatulence. She was given a yeast autolysate[6] for two months. At the end of this period stomatitis and glossitis developed. The abdominal discomfort was still present, and she was admitted to the hospital.

Physical Examination.—The patient was well developed and well nourished. The only abnormalities noted were several aphthous ulcers on the buccal mucosa, with injection and redness of the entire oral mucous membrane. The tongue was red, raw and obviously sore.

Laboratory Examinations.—Gastric analysis showed no free hydrochloric acid even after the injection of histamine. Studies of the blood showed: erythrocytes, 3,600,000; hemoglobin, 91 per cent; color index, 1.27, and mean corpuscular volume, 115 cubic microns.

Course.—The patient was given 10 cc. of liver extract by intramuscular injection daily for four days. The glossitis, stomatitis and flatulence disappeared. Since discharge she has remained symptom free for ten months while taking a single intramuscular injection of 10 cc. of liver extract once every two weeks.

Summary: This group consists of fourteen patients who gave a history of repeated attacks of diarrhea. All except one had had soreness of the mouth. None of them had diarrhea on admission to the hospital, but eleven had symptoms referable to the gastro-intestinal tract. This is a group of patients with "incomplete sprue," that is, patients observed between attacks of the disease. Two typical cases are presented, both of which demonstrate the effect of liver extract on the minor gastro-intestinal symptoms.

Roentgenographic Picture (Table 6)

Morphologic alterations in the small intestines of patients with sprue, as demonstrated by roentgenograms, have been described in a previous paper.[7]

Furthermore, a return toward normal form and function has been shown by roentgenographic means to accompany the cessation of symptoms following the injection of liver extract.[8] The chief morphologic alterations have been summarized as follows: (1) a variation in the caliber of the intestinal segments; (2) a distortion of the mucosal pattern, usually of the jejunum but often of the duodenum and ileum as well, and (3) a segmental distribution of the barium, which is probably produced by abnormal motility of portions of the small intestine.

Among these alterations the variation in the caliber of the intestinal loops and the distortion of the mucosal pattern are the most characteristic changes in sprue. It is not assumed that these changes are pathognomonic of the disease; they are merely presented as observations which were made during the study of a large group of patients

TABLE 6

Roentgen Studies of Small Intestine

Case	Variation in Caliber		Changes of Mucosal Pattern		Segmentation
	Jejunum	Ileum	Jejunum	Ileum	
Group A					
1	+	+	+	+	+
3	..	+	+	+	..
4	+	+	+	+	+
5	+	+	+	+	+
6	+	+	+	+	+
7	+	+	+	+	+
8	+	+	+	+	+
9	+	+	+	..	+
10	..	+	+	+	+
Grand total (9 cases)	7	9	9	8	8
Group B					
11	..	+	+	..	+
12	..	+	..	+	+
13	..	+	+	+	..
14	+	+	+	+	+
15	+	+	+	..	+
16	..	+	+	+	+
17	..	..	..	..	+
18	..	+	..	..	+
19	..	+	..	+	+
Grand total (9 cases)	2	8	5	5	8
Group C					
20	+	+	+	..	+
21	..	+	+	..	..
23	+	+	+	..	+
25	..	+	..	+	..
26	+	+	+	+	+
27	..	+	..	..	+
28	..	+	..	+	..
29	+	..	+	..	..
30	..	+	..	+	+
32	..	+	..	+	+
33	..	+	..	..	+
Grand total (11 cases)	4	10	5	5	7
Series total (29 cases)	13	27	19	18	23

with sprue. Snell and Camp[9] have reported similar roentgenographic changes in cases of idiopathic steatorrhea. Recently Ravdin, Pendergrass and their associates[10] have studied the effect of various types of food on the roentgenographic pattern of the small intestine in normal persons. As these authors pointed out, it is of interest that after an olive oil meal the intestine shows changes much like those seen in patients with various forms of steatorrhea. Since poor fat absorption is a characteristic of sprue, it is possible that some of the changes observed in the pattern of the small intestine of patients with sprue are associated with the presence of an abnormal amount of fat in the bowel. Few adequate roentgenographic studies of the small intestine in disease states have been carried out; it is therefore difficult to compare the findings in sprue with those present in other intestinal disorders.

The method of roentgen examination used in this study was that previously described;[11] in the more recent studies the amount of barium sulfate used was reduced from 4 to 2 ounces (125 to 62 Gm.) at the suggestion of Dr. Golden, of the College of Physicians and Surgeons of Columbia University. Table 6 summarizes the principal alterations which were seen in the roentgenograms of twenty-nine patients with sprue, and illustrations from several cases are presented.

Variation in Caliber of the Intestinal Loops. Variation in the caliber of the intestinal loops occurred most frequently in the ileum, somewhat less frequently in the jejunum and rarely in the duodenum. The greatest variations in caliber were noted in studies of patients with severe sprue. Here the intestinal loops often appeared to be atonic; they were frequently dilated to such a degree that the diameter approached that of the large bowel (fig. 3 *A*). In other instances the deviation from the normal caliber of the small intestine was less marked, yet there was sufficient variation in caliber to suggest spasticity in certain areas and dilatation in others (figs. 3 *B* and 5 *A*). In the severe form of the disease these changes were found in the jejunum and ileum; in the milder form the ileum alone was involved.

Distortion of the Mucosal Pattern. Distortion of the mucosal pattern was commonly noted in the jejunum and ileum, less frequently in the duodenum. The most striking changes occurred in the jejunum; in the cases of milder involvement the delicate feathery pattern of the normal jejunum (fig. 4 *A*) was replaced by a much coarser pattern (fig. 4 *B*), whereas in many of the cases of more severe involvement the valvulae conniventes were markedly thickened and widely separated, giving rise to a picture not unlike that of the normal colon (fig. 3 *A*).

All the patients of group A showed distortion of the mucosal pattern in the jejunum, and all but one showed similar alterations in the ileum.

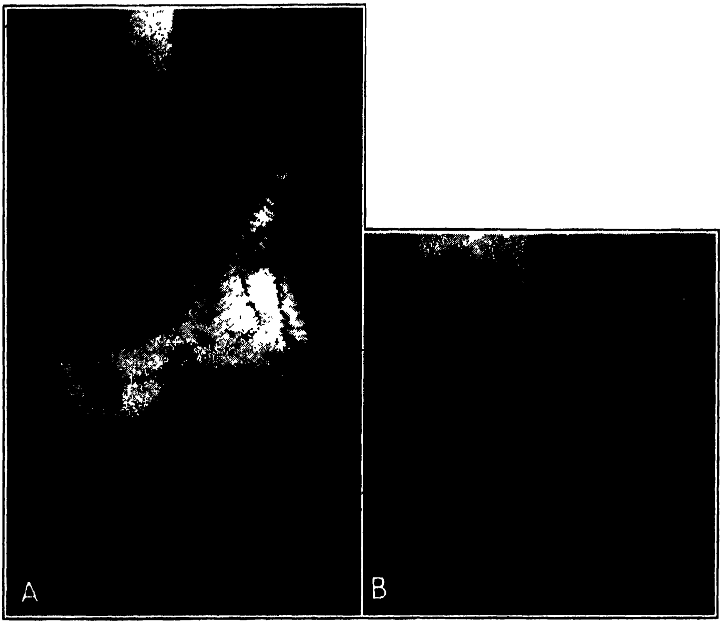

FIG. 3.—*A*, severe sprue (case 4). Note the abnormally dilated small intestine, with striking deviation of the mucosal pattern from normal. *B*, mild sprue (case 29). Note the variation in the caliber of the intestinal loops.

In groups B and C an equal number of patients showed changes in the jejunum and in the ileum.

Segmentation.—Isolated segments of the intestine frequently presented a sausage-like appearance, which was usually observed in the middle and lower portions of the ileum (fig. 5 *A*). This segmental distribution or pocketing of the barium was considered to be due to an irregularity of the movement of the contrast medium. The segmentation was interpreted as caused by alternating areas of spasticity and dilatation of the intestine. This type of segmentation was observed in all patients of groups A and B except one and in some patients of group C. Such a patchy distribution of barium has been observed in other conditions associated with diarrhea.[12]

In addition to the three most common alterations of the small intestine just described, certain other abnormalities were noted. In twelve instances the barium advanced through the small intestine in a divided column (fig. 5 *B*), whereas normally the column is continuous. This separation of the opaque material into two or more columns suggested that the progression was temporarily arrested at one or more points along the intestinal tract. A terminal ileum which seemed to be straighter and more rigid than normal was observed in eleven cases. The roentgenographic picture of the colon was essentially normal in every instance in which a barium enema was given.

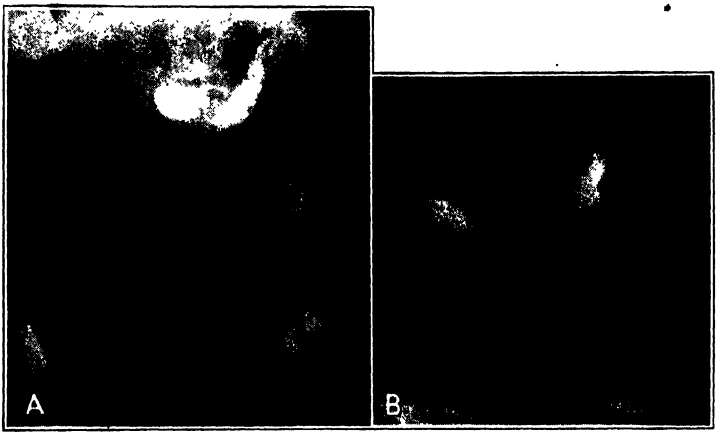

FIG. 4.—*A*, mild sprue (case 32). The essentially normal jejunum should be compared with the appearance in figures 3 *A* and *B* and 4 *B*, mild sprue (case 29). Note the distortion of the mucosal pattern of the jejunum.

Summary of Roentgen Findings.—A distortion of the mucosal pattern and a variation in the caliber of the intestinal loops were the most significant changes observed roentgenographically in the small intestines in twenty-nine cases of sprue. All the patients showed these changes in various degrees. In general, the more severe the disease, the more pronounced the morphologic alterations. The roentgenographic findings should not be regarded as pathognomonic of sprue, as no comparisons can be made until more patients with other intestinal disorders have been studied by the same technic. Nevertheless, a correlation appears to exist between the severity of the disease and the extent of the morphologic alterations. Furthermore, these changes can be shown to regress after the use of specific antisprue therapy.

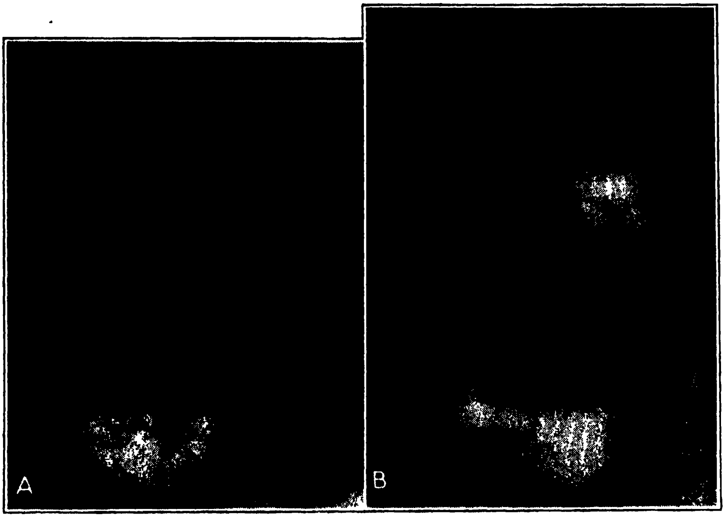

FIG. 5.—*A*, severe sprue (case 1). Note the variation in the caliber of the ileal loops and the segmental distribution of the barium in the ileum. *B*, note the separation of the barium meal into two columns (case 4).

Treatment

The results obtainable through the adequate treatment of sprue are convincingly illustrated by this series of thirty-three patients. For the purpose of discussion the therapy employed is most conveniently divided into three phases: (1) treatment before admission to the hospital, (2) treatment during hospitalization and (3) treatment following discharge from the hospital.

1. *Treatment Before Admission to the Hospital* (Table 7).—It is of interest to review briefly the various types of treatment administered to the patients of this series before they were admitted to this hospital. Table 7 summarizes the treatment and results under three separate headings; diet, liver extract administered orally and liver extract administered parenterally.

Of the twenty-seven patients for whom a dietary regimen had been prescribed, twenty-two had been given some form of diet for sprue, that is a diet high in protein and low in carbohydrate and fat. Six received no benefit from this diet, whereas sixteen showed distinct improvement early in the course of the disease. Six of the sixteen patients who improved had been specifically instructed to include whole liver in their diet. Once the symptoms of sprue had been controlled by strict dietary measures, the patients took liberties with their diet or abandoned it entirely. The result was a recurrence of gastro-intestinal and oral symptoms. When such relapses set in, the patients frequently found that diets which had induced remissions in the past were no longer effective. Case 12, described in detail previously, well illustrates such a sequence of events.

Seven patients had received liver extract orally at one time or another during the course of their illness. Four were temporarily improved, whereas three were unimproved.

Liver extract had been administered parenterally to twelve patients. Three showed distinct symptomatic relief. Six were temporarily benefited and, to judge from the subsequent course, would have continued to improve if the parenteral therapy had been continued. In three cases the symptoms were not alleviated, presumably because the amount of liver extract given was too small. For example, one patient (case 12) did well when he received an intramuscular injection of 3 cc. of liver extract every day, yet later he showed no response to an intravenous injection of 2 cc. once a week.

It is apparent that the great majority of the patients in this series had been inadequately treated before they were admitted to the hospital. Temporary remissions had been effected in many cases both by dietary measures and by liver extract, but therapy had not been sufficiently intensive to maintain the patients in good health.

2. *Treatment During Hospitalization* (Table 8).—The three chief therapeutic measures employed in the treatment of these patients in

TABLE 7

*Treatment before Hospitalization**

Case	Diet	Result	Liver Extract, Oral	Result	Liver Extract, Parenteral	Result
Group A						
1	Cooked liver and sour milk (S)	Temp.	3 vials q.d., 14 months	No imp.		
2	Eggs, milk and chopped meat; no starch or carbohydrate (S)	No imp.	....	....	2 cc. intramusc. q.d., 5 weeks	No imp.
3	Rice and few vegetables	No imp.				
4	Meat and eggs (S)	Temp.	6 vials q.d., 3 months: 9 vials q.d. 6 months	Temp.		
5	High protein, low carbohydrate and fat (S)	Temp.	....	....	2 cc. intramusc. twice a week	No imp.
6	Cereals, eggs, milk, fruit juices, bananas, cream, high protein and cooked liver (S)	Temp.				
7	a. Low residue	Temp.	Exact amount not known	No imp.	Intraven. injections for 3 weeks	Temp.
	b. High protein (S)	No imp.				
8	....	....	....	....	10 intramusc. injections; 4 weekly for short time	Temp.
9	High protein (S)	No imp.				
10	a. Eggs, milk and bananas (S)	Temp.				
	b. Brown bread and pineapple	Worse				

Group B

11	Coffee, cereal, bread, pork, chicken and some milk	No imp.				
12	Diet for sprue with liver (S)	Temp.	3 vials q.d.	No imp.	a. 3 cc. intramusc. q.d., 5 months	Imp.; tired of treatment
					b. 2 cc. intraven. weekly	No imp.
13	Low starch (S)	Temp.				
14	a. High protein, low fat and carbohydrate (S)	....	....	....	a. Campolon intramusc. q. 2 days, 2 weeks	No imp.
	b. Raw meat and bananas (S)	No imp.	....	....	b. 2 cc. intramusc. 4 q. week, 5 weeks	No imp.
	c. High carbohydrate, little fat or protein					
15	a. Liver, meat and fish (S)	Temp.				
	b. Fish, fruit, vegetables, potatoes, broths, custards and crackers	Temp.				
16	(S)	Temp.	....	....	10 cc. intramusc. twice a week	Imp.
17	Low residue	Slight, temp.				
18	a. 3 qts. of milk, grapefruit juice, rare beef and liver added (S)					
	b. Liver with milk and red meats (S)	Temp.				
19	Eliminated orange juice	Temp.				

* (S) signifies diet for sprue; temp., temporary improvement; imp., improvement; no imp., no improvement; intramusc., intramuscularly, and intraven., intravenously.

TABLE 7—*Concluded*

Case	Diet	Result	Liver Extract, Oral	Result	Liver Extract, Parenteral	Result
Group C						
20	Eliminated fatty and starchy foods (S)	Temp.				
21	....	....	....	....	a. 10 cc. intramusc. q. week	Temp.
					b. 2 cc. intramusc. twice a week	Temp.
22	(S)	No imp.	....	....	Small amount irregularly	Temp.
23	a. Skimmed milk and strawberries	No imp.				
	b. Fruits and vegetables with red meat (S)	Temp.				
24	(S)	No imp.	4 capsules q.d., 6 months	Temp.	39 cc. intramusc. in 2 weeks	Temp.
25	Rare scraped beef and Bulgarian buttermilk (S)	Temp.				
26	Fresh vegetables and meat (S)	Temp.	3 vials q.d., 3 months	Temp.		
27	High protein (S)	Temp.				
28						
29						
30	High meat diet with liver (S)	Temp.	....	....	Course of injections every spring and fall	Imp.
31	Low fat-low salt diet	No imp.				
32	....	....	2 courses of treatment	Temp.	4 intramusc. injections in 2 weeks	Temp.
33						

the hospital were: (*a*) rest in bed, (*b*) a diet for sprue and (*c*) liver extract given parenterally.

(*a*) Rest in Bed: Complete rest in bed was of course essential in the cases of severe sprue and also furnished a useful adjunct to the more specific forms of therapy in the cases of a milder degree of involvement. It was not uncommon to find that a patient required more intensive therapy when ambulatory than when at rest in bed. Undue fatigue appeared to play a prominent rôle in the precipitation of relapses.

(*b*) Diet for Sprue: The diet for sprue as used in this clinic was essentially high in protein and low in carbohydrate and fat. Emphasis was placed on the taking of large amounts of rare red meat. The dietary regimen is presented in table 9. Practically all the patients received this diet unless the severity of their condition made such a measure impossible. Then the patient was given nothing but ground meat and milk until he could partake of a more liberal fare.

Three patients in group C were treated with the diet for sprue alone and became practically symptom free on this regimen. One patient was not treated, as he was referred to this hospital for diagnostic study only. For the remaining twenty-nine patients the dietary treatment was not effective until it was supplemented with injections of liver extract. No patient with sprue of moderate or severe degree was seen for whom dietary treatment alone brought about complete amelioration of symptoms.

(*c*) Liver Extract: Unless otherwise specified, solution of liver extract for parenteral use, both intramuscular and intravenous, was prepared from liver extract[4] which met the standard given in "New and Nonofficial Remedies." The concentration of the solution for intramuscular injection was twice that of the solution for intravenous use. The solutions were made up so that 10 cc. of the intramuscular preparation or 20 cc. of the intravenous preparation contained the amount of extract derived from 50 Gm. of whole liver. The maximal and usual dose of the intramuscular extract given at one time was 10 cc.; of the intravenous extract, 20 cc. An intramuscular injection was sometimes productive of soreness and burning at the site of injection but was rarely followed by systemic reactions. The intravenous injections were occasionally followed by chills, fever, flushing, headache, nausea

TABLE 8

Treatment

Case	During Hospitalization				After Discharge		Total Period of Observation
	Diet	Liver Extract, Intramuscular	Liver Extract, Intravenous	Result	Liver Extract	Comments	
				Group A			
1	No bread or potatoes	20 cc. 1 day	....	....	10 cc. q. 2 weeks	Well	4½ yr.
		10 cc. q.d., 9 days	....	Improvement			
		2 cc. q.d., 6 days	....	Diarrhea			
		10 cc. q.d., 9 days	....	Improvement			
2	Sprue	10 cc. q.d., 3 days 10 cc. q. 2 weeks 10 cc. q. 10 days	....	Improvement	20 cc. q. month	Returned to Puerto Rico; symptom free	4½ yr.
3	Sprue	....	20 cc. q.d., 30 days	Improvement	10 cc. q. 2 weeks	Well	3½ yr.
4	Sprue	10 cc. q.d., 10 days	20 cc. q.d., 5 days	Improvement	10 cc. q. week 10 cc. twice a week	Discontinued liver; had relapse in 2 months; readmitted; now symptom free	3½ yr.
5	Sprue	10 cc. q.d., 4 days	20 cc. q.d., 10 days	Improvement	10 cc. q. 2 weeks	Symptom free	3 yr.
6	Sprue	10 cc. q.d., 30 days	20 cc. q.d., 20 days	Improvement temporarily	....	Died of tuberculous peritonitis	6 mo.
7	Sprue	....	20 cc. q.d., 5 days	Improvement	Irregular	Died of carcinoma of pancreas	1¾ yr.
8	Light	10 cc. q.d., 10 days	....	Improvement	10 cc. q. 2 weeks	Relief of symptoms	4 yr.
9	Sprue	....	20 cc. q.d., 13 days	Improvement	....	Discharged against advice; not followed	2 mo.
10	Sprue	10 cc. q. 2 days for 10 injections	....	Improvement	10 cc. q. week	Symptom free	1 yr.

			Group B				
11	Sprue	10 cc. q.d., 10 days	....	Improvement	10 cc. q. week	Well	1¼ yr.
12	Sprue	....	20 cc. q.d., 8 days	Improvement	20 cc. intravenously q. 7–10 days; twice weekly	Fairly well at first; symptom free on treatments twice weekly	2¼ yr.
13	Sprue	10 cc. q.d., 7 days	....	Improvement	10 cc. twice weekly	Not improved	2¼ yr.
14	Sprue	....	20 cc. q.d., 11 days	Improvement	10 cc. q. 10 days	Well	2¼ yr.
15	Sprue	5 cc. q.d., 5 days	20 cc. q.d., 8 days	Improvement	10 cc. q. 2 weeks	Returned to China; well	3¼ yr.
16	Sprue	....	20 cc. q.d., 5 days	Improvement	10 cc. q. week	Well	2⅓ yr.
17	Sprue	10 cc. q.d., 8 days	20 cc. q.d., 10 days	Improvement	....	Not followed in clinic	4 mo.
18	Sprue	10 cc. q.d., 7 days	....	Improvement	10 cc. q. 2 weeks	Returned to Puerto Rico; well	2¼ yr.
19	Sprue	....	20 cc. q.d., 20 days	Improvement	....	Died of sprue in another hospital without therapy	1 yr.
			Group C				
20	Sprue	....	....	....	....	Not treated in hospital	2 mo.
21	Sprue	3 cc.* q.d., 13 days	....	Improvement	10 cc. q. 2 weeks	Returned to Puerto Rico; well	4 yr.
22	Sprue	10 cc. q. week	....	Improvement	10 cc. q. week	Well	2 yr.
23	Sprue	10 cc. q.d., 5 days	....	Improvement	10 cc. twice weekly; 10 cc. q. week	Well	2¼ yr.
24	Sprue	10 cc. q.d., 6 days 10 cc. thrice weekly, 3 weeks	20 cc. q.d., 5 days	Improvement	10 cc. q. 2 weeks	Well; returned to China	1¼ yr.
25	Sprue	10 cc. for 4 injections	....	Improvement	10 cc. q. 2 weeks	Well	9 mo.
26	Light	10 cc. for 2 injections	....	Improvement	....	Advised to return to China; not followed	1 mo.

* Liver extract-Lederle (N. N. R.) was used.

TABLE 8—*Concluded*

Case	During Hospitalization				After Discharge		Total Period of Observation
	Diet	Liver Extract, Intramuscular	Liver Extract, Intravenous	Result	Liver Extract	Comments	
Group C—*Concluded*							
27	Sprue	....	....	Improvement	....	Well on diet	1 yr.
28	Sprue	....	....	Improvement	....	Well on diet	3 mo.
29	Sprue	10 cc. q.d., 8 days	....	Improvement	10 cc. q. 2 weeks	Well	1 yr.
30	Sprue	10 cc. q. week	....	Improvement	10 cc. q. week	Well	1 yr.
31	Sprue	10 cc. q. 2 days, 12 injections	....	Improvement	10 cc. q. 2 weeks	Well	1⅓ yr.
32	Sprue	10 cc. q.d., 4 days	....	Improvement	10 cc. q. 2 weeks	Well	1⅓ yr.
33	Sprue	....	....	Improvement	....	Fairly well on diet alone	6 mo.

or urticaria. No reactions so severe as to require cessation of treatment were observed.

The total amount of liver extract injected into a given person during the period of hospitalization depended on the severity of the symptoms, the grade of anemia and the rapidity of the response to therapy. All the patients in groups A and B and ten patients in group C received parenteral injections of liver extract during their stay in the hospital. The amount of liver extract given to patients of groups A and B was greater than that given to patients of group C, as the former had a more severe form of the disease. One patient (case 6) received daily injections for fifty days with only temporary relief of his symptoms; this patient died later of tuberculous peritonitis. Another patient (case 3) received daily intravenous injections for thirty days. The majority of patients in the first two groups required daily injections over periods of from five to twenty days, whereas shorter courses of parenteral therapy usually brought about complete relief of symptoms in the patients in group C. The importance of intensive liver extract therapy in the treatment of cases of severe sprue has already been emphasized.[18] Too much stress cannot be laid on the fact that much larger amounts of liver extract are generally necessary to control the symptoms of sprue than are required to induce a remission in a case of severe pernicious anemia.

The alleviation of the various symptoms of sprue with combined dietary and liver extract therapy was on the whole phenomenal. The gastro-intestinal symptoms—diarrhea, flatulence, anorexia and nausea—cleared up entirely in all but two patients treated in the hospital. When these symptoms were under control, the patients began to gain weight at a rate which was often astounding in view of the dietary restriction of carbohydrate and fat. Soreness of the tongue and mouth disappeared, aphthous lesions healed and in those patients who showed papillary atrophy regeneration of the papillae took place.

No less striking was the improvement in the blood picture of those patients who had anemia. The red blood cell count, hemoglobin value and white blood cell count all returned toward normal levels, as did the mean corpuscular volume and the color index.

Thus it is apparent that the various clinical manifestations of sprue, both mild and severe, were dramatically alleviated in the great major-

TABLE 9

Diet for Sprue

Breakfast

Toast, 1 thin slice with a small piece of butter
Fresh fruit, large serving
Eggs, 2, prepared in any manner desired
Milk, at least 1 large glass (this may be heated and used in place of cream for coffee or may be made into cocoa)

Luncheon

Meat, large serving (¼ pound at least)
Vegetables, 2, or 1 vegetable and salad
Milk puddings, gelatin, ice-cream or fresh fruit
Crackers (2 only) with cheese
Milk, 1 glass

Dinner

Fruit cocktail or tomato juice
Beef broth or any cream soup (make cream soups with milk instead of cream and omit butter)
Meat, large serving (¼ pound at least)
Vegetables, 2 at least
Desserts, as for lunch
Milk, 1 glass

Egg-nogs, milk or fresh fruit may be eaten between meals

The diet should consist largely of protein:
  Meat: Meat should always be lean and rare
    Beef, lean, either roast or steak
    Lamb, lean, either roast or chops
    Ham, lean
    Liver, tongue, heart, kidney or sweetbreads
    Fish, any fresh fish
    Chicken
    Lobster, crab or oysters
  Milk and milk products: milk, buttermilk and American and cottage cheese
  Eggs: prepared in any manner desired

All fatty foods should be avoided: butter, cream, lard, olive oil and all oil salad dressing, avocado pears, pie, pastry and all foods fried in fat

All starchy foods should be avoided: potatoes, beans, corn, bread, cereals, cake and candy.

ity of cases by the institution of adequate therapy. In the cases of more severe involvement therapy was necessarily intensive at first, whereas in the cases of milder involvement several injections of liver extract, or at times dietary measures alone, sufficed to render the patients symptom free.

(*d*) Adjunctive Therapeutic Measures: In addition to rest in bed, a diet for sprue and liver extract, certain other therapeutic measures were indicated in a small percentage of patients. These measures were blood transfusion, calcium salts and iron. If the anemia is so severe as to jeopardize the patient's life, blood transfusion should be given. Only one patient in this series (case 2) required transfusion. Calcium salts should be administered both orally and parenterally to patients with tetany, muscle cramps or osteoporosis. Two patients (cases 6 and 7) who had tetany were given intravenous injections of calcium chloride during the acute stage; later they were given calcium gluconate by intramuscular injection. Muscle cramps were relieved by the oral administration of calcium chloride in case 4. The addition of intramuscular injections of calcium to liver extract therapy afforded more complete relief from gastro-intestinal symptoms in a few cases. Occasionally the anemia of sprue was microcytic, or the blood picture changed from a macrocytic to a microcytic type after treatment with liver extract. In such cases iron, in the form of ferrous sulfate or iron and ammonium citrates, was given as a supplement to liver extract therapy.

3. *Treatment Following Discharge from the Hospital* (Table 8).—Perhaps more important than the alleviation of the acute symptoms is the maintenance of patients with sprue in good health over a prolonged period. Data are available for twenty-nine of the thirty-three patients over periods of from three months to four and one-half years after discharge.

Three patients of this series have died. One patient (case 6) died of tuberculous peritonitis after six months, and another (case 7) died of carcinoma of the pancreas after twenty-one months. It is of interest that the symptoms of both patients were temporarily relieved by antisprue therapy. One patient (case 19) died of sprue in another hospital, where he was not given specific therapy.

One patient (case 13) continued to have attacks of diarrhea, cramps and flatulence in spite of diet for sprue and injections of liver extract twice a week. The addition of liver extract orally and injections of calcium gluconate alleviated the symptoms to a certain extent but failed to afford complete relief. For this reason the patient was readmitted to the hospital for further study. No amebas were found in the stools, and culture revealed no pathogenic organisms. With rest in bed in addition to dietary and liver extract therapy, the diarrhea and cramps ceased, only to recur when the patient resumed her secretarial work. This is the only patient with tropical sprue whose symptoms have not been controlled by the antisprue regimen which has been described.

Another patient (case 33) has been only "fairly well" on the diet for sprue alone. To judge from the course of the other patients studied it is possible that an occasional injection of liver extract would render this patient free from symptoms.

The remaining twenty-four of the twenty-nine patients for whom adequate follow-up data are available have remained practically symptom free with continued antisprue therapy. Five of these patients have returned to tropical countries without a recurrence of symptoms. Two of the twenty-four have remained essentially well on the diet for sprue alone. The other twenty-two patients have required injections of liver extract at regular intervals. One patient is given an intravenous injection of 20 cc. twice a week and another an intramuscular injection of 10 cc. twice a week. Seven patients receive a single intramuscular injection of 10 cc. once a week, twelve patients receive an intramuscular injection of 10 cc. every two weeks and one patient remains symptomatically well with one intramuscular injection a month.

The interval that is allowed to elapse between injections of liver extract must be ascertained for each patient. Injections should be given at sufficiently frequent intervals to prevent the recurrence of gastro-intestinal symptoms. One patient (case 1) who has been followed for four years may be taken as an illustration of this principle. During the first year after discharge from the hospital this patient suffered from recurrent gastro-intestinal symptoms which began about ten days after an injection of liver extract; therefore she was treated once a week. More recently she has found that she remains free from symptoms for nearly three weeks after an injection; accordingly the interinjection interval has been increased to two weeks.

The intimate relationship that exists between the injection of liver extract and the cessation of gastro-intestinal symptoms has been recognized by many patients. A patient who has symptoms as a result of transgressing the safe interval between treatments usually experiences complete relief from these symptoms within twenty-four hours after an injection of liver extract. Furthermore, a number of patients have found through repeated trials that soon after a treatment with liver extract they may take many liberties with their diet with impunity, whereas during the latter part of the interinjection interval they must adhere strictly to the diet for sprue in order to prevent the recurrence of gastro-intestinal symptoms.

Both mental and physical fatigue were often responsible for the onset of symptoms in these patients. During periods of stress the frequency of injections had to be increased. Case 30 serves to illustrate just such a situation. This patient suffered from a "nervous breakdown" several weeks after she had been rendered free from symptoms of sprue by specific therapy. During a period of two weeks she was upset mentally, ate little and received no liver extract. At the end of this period she had a typical relapse of sprue with diarrhea, flatulence and a sore red tongue. These symptoms cleared up rapidly when treatment with liver extract was resumed.

The question naturally arises as to whether the patient with sprue can ever abandon specific therapy entirely without suffering a relapse. All that can be said in answer to this question is that those patients who have voluntarily discontinued therapy have suffered relapses; this occurred, for example, in case 4, described in detail under group A. The majority of these patients have been unwilling to abandon their therapeutic regimen for fear of suffering a relapse. It is evident that the well-being of a patient with sprue is dependent on the regulation and maintenance of this specific regimen. The amount of liver extract which is necessary to prevent the recurrence of gastro-intestinal symptoms is far greater than that which is required to maintain normal blood values. Hence the basic principle in the treatment of sprue is the administration of an adequate amount of liver extract at sufficiently frequent intervals to prevent the recurrence of abdominal discomfort, flatulence and diarrhea.

SUMMARY AND CONCLUSIONS

A series of thirty-three patients with sprue has been studied. Twenty-nine of these patients have been followed in the clinic after discharge from the hospital. The patients have been divided into three groups on the basis of certain symptoms and clinical findings at the time of entry into the hospital. Ten patients presented the classic picture of severe sprue with diarrhea and anemia. Nine patients had diarrhea but no anemia, while the remaining fourteen complained only of abdominal discomfort and flatulence. The symptomatology, the blood findings and the results of gastric analyses for these patients have been presented in detail, together with abstracts of illustrative cases.

Roentgen study of the small intestine was made for twenty-nine of these patients, and morphologic alterations of the small intestine were observed in all, these changes varying directly with the severity of the disease. The most significant abnormalities were a distortion of the mucosal pattern and a variation in the caliber of the intestinal loops. These roentgenographic changes are not regarded as being pathognomonic of sprue, since not enough studies of the small intestine have been made in cases of other gastro-intestinal disorders for purposes of comparison.

Treatment before, during and after hospitalization has been discussed in detail. The great majority of the patients experienced complete symptomatic relief once adequate therapy had been instituted. Moreover, these patients have been maintained in excellent health over long periods by means of a relatively simple therapeutic regimen. Five patients have returned to tropical countries without recurrence of symptoms. It must be emphasized that no patient has discontinued specific therapy entirely without suffering a relapse. The maintenance of a diet for sprue in addition to liver extract therapy gives a patient more complete relief of gastro-intestinal symptoms than does liver extract given parenterally alone. The frequency of injections of liver extract must be worked out for each patient, the prevention of intestinal dysfunction serving as a guide to the amount of therapy to be given.

1. Castle, W. B.; Rhoads, C. P.; Lawson, H. A., and Payne, G. C.: Etiology and Treatment of Sprue, Arch. Int. Med. **56**:627 (Oct.) 1935.

2. Fairley, N. H.: Tropical Sprue with Special Reference to Intestinal Absorption, Brit. M. J. **1**:1176, 1936; Tr. Roy. Soc. Trop. Med. & Hyg. **30**:9, 1936.

3. Reed, A. C.: Sprue, a Clinical Summary, Am. J. Trop. Med. **16**:499, 1936.

4. Liver extract-Lilly (N. N. R.) was used.

5. Liver extract-Parke, Davis & Co. (N. N. R.) was used.

6. The preparation used was vegex.

7. Mackie, T. T.; Miller, D. K., and Rhoads, C. P.: Sprue: Roentgenologic Changes in the Small Intestine, Am. J. Trop. Med. **15**:571, 1935.

8. Miller, D. K., and Rhoads, C. P.: The Effect of Liver Extract on the Small Intestine of Patients with Sprue, Am. J. M. Sc. **191**:453, 1936.

9. Snell, A. M., and Camp, J. D.: Chronic Idiopathic Steatorrhea: Roentgenologic Observations, Arch. Int. Med. **53**:615 (April) 1934, Proc. Staff Meet., Mayo Clin. **10**:177, 1935.

10. Ravdin, I. S.; Pendergrass, E. P.; Johnston, C. G., and Hodes, P. J.: The Effect of Foodstuffs on the Emptying of the Normal and Operated Stomach and the Small Intestinal Pattern, Am. J. Roentgenol. **35**:306, 1936. Pendergrass, E. P.; Ravdin, I. S.; Johnston, C. G., and Hodes, P. J.: Studies of the Small Intestine: II. The Effect of Foods and Various Pathologic States on the Gastric Emptying and the Small Intestinal Pattern, Radiology **26**:651, 1936.

11. Mackie, T. T., and Pound, R. E.: Changes in the Gastro-Intestinal Tract in Deficiency States, with Special Reference to the Small Intestine: A Roentgenologic and Clinical Study of Forty Cases, J. A. M. A. **104**:613 (Feb. 23) 1935.

12. Mills, R. W.: X-Ray Evidence of Abdominal Small Intestinal States Embodying an Hypothesis of the Transmission of Gastro-Intestinal Tension, Am. J. Roentgenol. **9**:199, 1922.

13. Rhoads, C. P., and Miller, D. K.: Intensive Liver Extract Therapy of Sprue, J. A. M. A. **103**:387 (Aug. 11) 1934.

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CONGO RED IN THE TREATMENT OF PERNICIOUS ANEMIA AND SPRUE

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In 1933, Massa and Zolezzi,[6a] at the University of Parma Medical Clinic, announced the successful treatment of 2 cases of pernicious anemia with intravenously administered Congo red. A year later they reported favorable results in a larger series of cases.[6b] In 1935, Mermod and Dock[5] apparently confirmed the observations of the Italian authors. Since Congo red is a synthetic colloidal dye with a known chemical formula, it seemed most important to test further the use of this compound in the treatment of pernicious anemia.

Massa and Zolezzi employed a 0.5% solution of Congo red (Grübler) made up in 0.5% saline solution. This solution was warmed almost to boiling, allowed to stand 24 hours, and then filtered through paper. The filtrate was placed in ampules and sterilized by autoclaving for 10 minutes at $\frac{3}{4}$ to 1 atmosphere. Injections of 20 cc. of this solution were given every 1 to 2 days for 5 to 6 days followed by a rest period of several days. Of fifteen cases treated with Congo red, as far as can be ascertained from the brief case reports, the majority satisfied the usual criteria for the diagnosis of pernicious anemia, *i.e.*, a macrocytic hyperchromic anemia, hyperbilirubinemia, and achylia gastrica. Of the 15 cases, 3 may be dismissed at once as they were given a short course of liver therapy prior to the injections of Congo red. The authors felt that the continued improvement on Congo red pointed to a specific therapeutic effect of the dye; however, as it is well established that small amounts of liver may produce complete and prolonged remissions in pernicious anemia, it would be impossible to evaluate the efficacy of Congo red in these 3 cases. Of the remaining 12 treated with Congo red alone, 9 had complete blood remissions, whereas 3 failed to respond at all. After receiving a fair trial of the dye, these latter 3 were treated energetically with liver extract to which they responded favorably. Reticulocytes were followed in 5 of the 9 cases which showed remissions on Congo red alone; in these 5 cases reticulocyte peaks of 10 to 25% occurred after the eighth or ninth injection. All of the patients treated successfully with Congo red remained clinically well for the several months of observation following treatment. In an attempt to explain the therapeutic effect of Congo red, Massa and Zolezzi suggested that the dye prevented hemolysis by blocking the reticulo-endothelial cells.

Mermod and Dock reported that they had confirmed the results of Massa and Zolezzi by successfully treating 2 cases of mild pernicious anemia with intravenous Congo red. These authors employed a 1.5% solution of the dye in 6% glucose solution. One patient received 60 cc. of this solution in 5 days, the other 90 cc. in 10 days. Both patients showed a rise in reticulocytes and a fall in serum bilirubin. As soon as the reticulocyte peak was passed, the treatment was changed to liver extract. It is therefore open to question whether complete blood remissions would have followed the reticulocyte crises if Congo red therapy had been continued. Mermod and Dock point to the fact that Congo red is notably effective in neutralizing a variety of toxins and suggest that "in pernicious anemia the dye assists in the detoxification of substances, probably enterogenous in origin, which are hemolytic."

A brief abstract of the results obtained in the treatment of pernicious anemia with Congo red at our clinic has recently appeared.[1] It is the purpose of the present report to give these results in more detail with some additional data.

Because of the difficulty in obtaining suitable cases of pernicious anemia in relapse, the problem was at first attacked from a somewhat different angle. Proven cases of pernicious anemia, which were in a remission as the result of liver extract therapy, were selected from the outpatient clinic. Weekly intravenous injections of Congo red (Grübler) solution were substituted for intramuscular liver extract injections.* A 0.5% solution of the dye in 0.5% saline, prepared exactly according to the method of Massa and Zolezzi, was employed in all cases. Only patients without evidence of combined system disease were selected. Six such patients were treated with Congo red for periods varying from 9 weeks to 6 months. The blood changes are illustrated by Charts I to VI and summarized in Table 1. It is evident that 5 of the 6 patients in this group evinced a marked tendency to relapse as shown by falling red count and hemoglobin as well as by rising mean corpuscular volume and color index. In 4 of the cases distinct symptoms of relapsing pernicious anemia appeared concomitant with the characteristic blood changes. The symptoms disappeared rapidly and the blood levels soon returned to normal when intramuscular liver extract therapy was resumed. These relapses during Congo red therapy are the more significant in that no attempt was made to control the diets of these patients, who had been previously trained to eat plenty of meat and other foods rich in Castle's extrinsic factor. As it is not unusual for pernicious anemia patients to maintain remissions for 6 months or longer without any specific therapy, it would certainly appear that weekly injections of Congo red completely fail to increase their chances of maintaining a remission. The criticism may be raised that these patients were not given sufficient Congo red and that larger or more frequent injections might have succeeded where the selected dosage

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* Liver Extract Lilly N.N.R. was used throughout this study. The solution for intramuscular injection was made up so that 1 cc. of solution contained the amount of extract derived from 5 gm. of whole liver.

TABLE 1

Blood Changes during Congo Red Therapy

Case No.	Patient	Duration of ℞ (wks.)	Total Congo red (cc.)	R. B. C. (millions)		Hb (%)		M.C.V. (μ^3)		C.I.	
				Before	After	Before	After	Before	After	Before	After
1	C. T.	9	160	4.09	2.90	90	77	95	104	1.10	1.32
2	C. M.	15	260	4.36	2.72	104	78	103	115	1.19	1.44
3	J. B.	18	360	4.36	2.66	105	74	104	112	1.20	1.39
4	I. F.	24	480	4.47	3.17	95	87	93	116	1.06	1.37
5	F. A.	25	500	4.97	1.99	101	63	92	132	1.02	1.58
6	M. A.	25	500	4.01	3.94	88	93	102	107	1.10	1.18
	Aver.	19.3	377	4.38	2.90	97	79	98	114	1.11	1.38
	Change			−1.48		−18		+16		+0.27	

failed. This is a valid objection, and it would not be safe to conclude from this experiment alone that Congo red is ineffective in pernicious anemia. The conclusion is justified, however, that weekly injections of Congo red did not furnish a practical substitute for liver extract in this group of patients, all of whom could be maintained in a complete remission by a single injection of liver extract once every 2 to 3 weeks.

More recently the opportunity arose to study the effect of Congo red in 2 cases of pernicious anemia in relapse. These patients were admitted to the ward and placed upon a diet low in Castle's extrinsic factor for some time before Congo red therapy was instituted. This procedure eliminated any possibility of a dietary effect and at the same time established a reticulocyte baseline. In the first patient (Case 7, Chart VII), daily intravenous injections of Congo red over a 2-week period failed to produce either subjective improvement or an increase in the reticulocytes, yet a subsequent small dose of liver extract gave rise to a satisfactory reticulocyte response at the expected time with the associated subjective improvement. The second patient (Case 3, Chart III), 1 of the cases which had relapsed while receiving weekly injections of Congo red, was admitted to the ward in order to see whether intensive Congo red therapy would succeed where weekly injections had failed. This patient, given daily injections of Congo red over a 3-week period, showed a minimal reticulocyte rise to 4% on the ninth day. Since there was no subjective improvement or change in the blood levels during the 3-week period this slight reticulocytosis was most likely a non-specific response. Thus in 2 cases of pernicious anemia in relapse, daily intravenous injections of Congo red completely failed to produce clinical or hematologic improvement.

Two cases of sprue were treated with Congo red in order to determine whether the dye might exert a therapeutic effect in this disease so similar to pernicious anemia in its response to liver extract. The first patient (Case 8) had been maintained free of symptoms over a long period on a sprue diet supplemented with a single injection of liver extract every 2 to 3 weeks. The diet was kept constant and weekly injections of 20 cc. of the Congo red solution were substituted for the liver extract régime. Within 4 weeks of the last liver extract injection the patient developed nausea and diarrhea, which became so severe that after 6 weeks the dye was discontinued and liver-extract therapy renewed with almost immediate alleviation of symptoms. The second patient (Case 9) was a known sprue patient suffering from diarrhea as a result of lapsing liver extract treatment for several weeks. This patient was brought into the hospital; in spite of daily injections of 20 cc. of the Congo red solution for 1 week, the diarrhea persisted and an aphthous stomatitis developed. Both the diarrhea and the stomatitis cleared up rapidly when liver extract injections were resumed.

Case Reports. CASE 1.—C. T., female aged 42 (Chart I). Onset of fatigue, yellow pallor, and sore tongue in 1929. Remission on liver extract shortly after onset. Relapsed when liver was discontinued. First came to clinic May, 1933.

Physical Examination. Lemon-yellow pallor. Marginal papillary atrophy of tongue. Palpable spleen. *Blood:* R.B.C. 1,800,000; Hgb. 43%; C.I. 1.19. *Gastric Analysis:* No free HCl, even after histamine. *Course:* Patient was treated with intravenous liver extract at first; later 10 cc. intramuscular liver extract every 2 weeks. Blood levels rose to normal and remained there. On September 30, 1935, R.B.C. 4,090,000; Hgb. 90%; M.C.V. 95; C.I. 1.10. On this date therapy changed to 20 cc. of Congo red solution once a week. At the end of 9 weeks R.B.C. had dropped to 2,900,000 and Hgb. to 77%; patient weak and tired, tongue very sore. Congo red discontinued and liver extract resumed with rapid symptomatic and hematologic improvement.

CASE 2.—C. M., male aged 60 (Chart II). In 1929 onset of fatigue, anorexia, sore tongue, paresthesias in legs, and anemia. Temporary improvement on oral liver; incomplete response to small doses of parenteral liver extract. February, 1933, admission to the hospital. *Physical examination:* Lemon-yellow pallor, marked papillary atrophy of tongue. Palpable spleen. Slightly diminished vibratory sense in legs. *Blood:* R.B.C. 960,000; Hgb. 24%; C.I. 1.26; W.B.C. 2250. *Gastric analysis:* No free HCl even after histamine. *Bone marrow:* Typical of pernicious anemia. *Course:* Remission on intravenous liver extract. Maintained in good health with blood at normal levels on 10 cc. of liver extract intramuscularly every 2 weeks. On October 3, 1935, R.B.C. 4,360,000; Hgb. 104%; M.C.V. 103; C.I. 1.19. Therapy changed to 20 cc. Congo red solution intravenously once a week. Glossitis and weakness developed. After 15 weeks R.B.C. had fallen to 2,720,000 and Hgb. to 78%. Congo red discontinued and intramuscular liver extract resumed with rapid remission.

CASE 3.—J. B., male aged 40 (Chart III). History of onset of weakness, fatigue, anorexia, sore tongue, and yellow pallor in 1928. Diagnosis of pernicious anemia was made and patient improved on liver therapy. Treatment was irregular and he suffered several relapses. First seen at this hospital June, 1935,

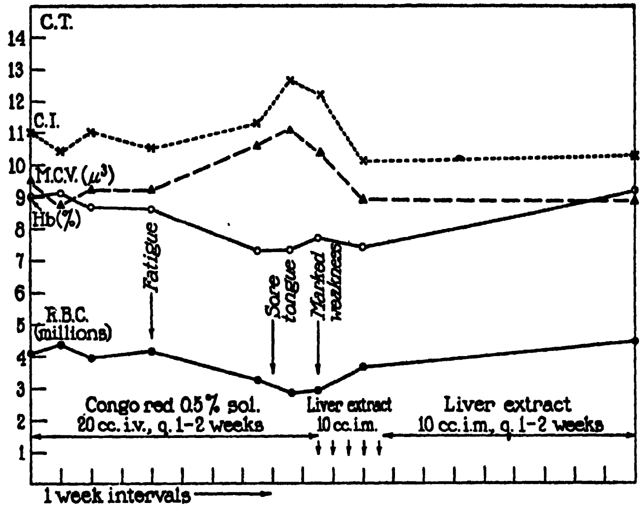

Chart I.—Case 1. Pernicious anemia. Rapid relapse during 9-week period of Congo red therapy.

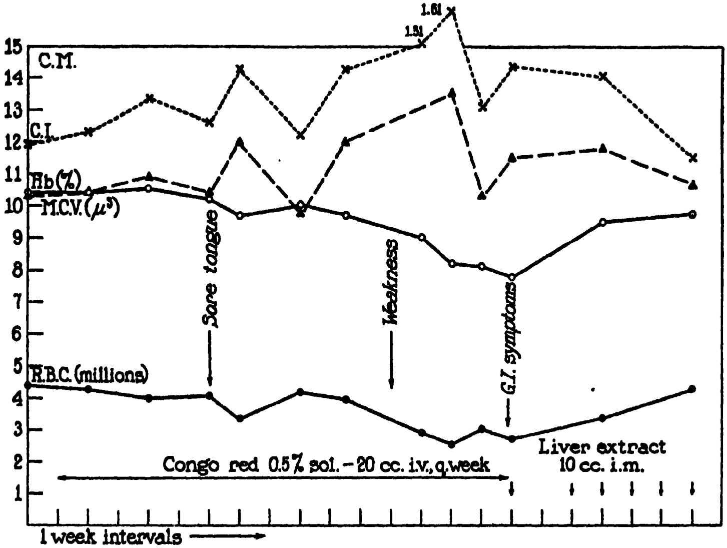

Chart II.—Case 2. Pernicious anemia. Relapse during 15-week period of Congo red therapy.

with R.B.C. 3,500,000 and Hgb. 94%. Liver extract 10 cc. given intramuscularly every 2 weeks; on this régime he felt very well; R.B.C. 4,360,000 and Hgb. 105%, with M.C.V. 104 and C.I. 1.20 in October, 1935, at which time therapy was changed to 20 cc. of Congo red solution intravenously once a week. This therapy was kept up for 18 weeks; during this period R.B.C. and Hgb. fell; M.C.V. and C.I. rose. He complained of increasing fatigue, exertional dyspnea, and finally soreness of tongue. He was then admitted to the hospital and placed on a meat-free diet. *Physical examination:* Moderate pallor. Redness of tongue. Slight diminution of vibratory sense in feet. *Blood:* R.B.C. 2,660,000; Hgb. 74%; M.C.V. 112; C.I. 1.39; Icteric Index 8; W.B.C. 6650. *Gastric analysis:* No free HCl even after histamine. *Course:* After 3 weeks on meat-free diet R.B.C. 2,000,000 and Hgb. 60%; icteric index 15. Daily intravenous injections of 20 cc. of Congo red solution were then given for 3 weeks. On 9th day reticulocytes had reached a low peak of 4% and then fell to original level below 2%. There was a slight fall in R.B.C. and Hgb. during the 3-week period and serum bilirubin remained elevated; the patient experienced no subjective improvement. Intramuscular liver extract therapy was subsequently renewed and blood levels rapidly returned to normal.

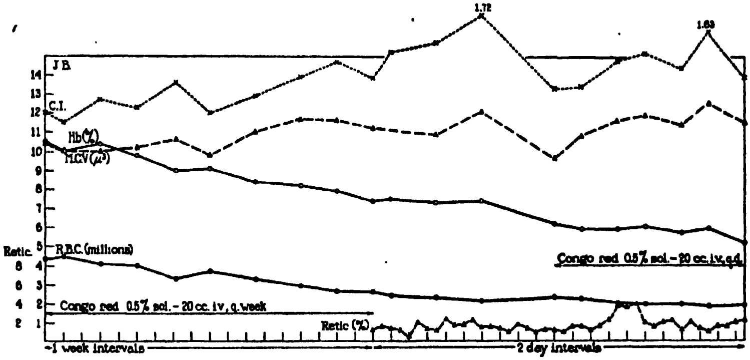

CHART III.—Case 3. Pernicious anemia. Relapse during 4-month period of weekly injections of Congo red. Failure to improve on daily injections of Congo red over 3-week period.

CASE 4.—I. F., female aged 39 (Chart IV). In 1934 onset of weakness, dizziness, pallor, anorexia, loss of weight. R.B.C. down to 1,000,000. Remission on liver extract but relapse with sore tongue and paresthesias when therapy was discontinued. Admission to the hospital July, 1935. *Physical examination:* Pallor. Slight icterus. Moderate papillary atrophy. Spleen just palpable. No neurologic disturbance. *Blood:* R.B.C. 2,080,000; Hgb. 58%; M.C.V. 121; C.I. 1.45; W.B.C. 5600. *Course:* Blood levels rose to normal on intramuscular liver extract and remission was maintained with a single injection of 10 cc. liver extract every 2 weeks. In September, 1935, R.B.C. 4,470,000; Hgb. 95%; M.C.V. 93; C.I. 1.06. Therapy changed to 20 cc. of Congo red solution once a week. After 24 weeks R.B.C. 3,170,000 and Hgb. 87%; the patient complained of weakness and sore tongue. Liver extract resumed with rapid improvement and 6 months later she is quite well with normal blood levels on a single injection of liver extract every 2 weeks.

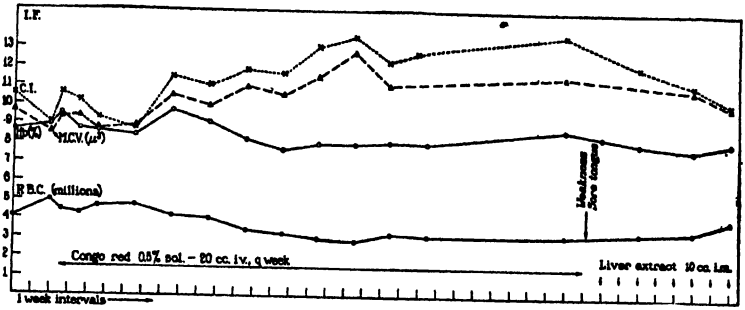

Chart IV.—Case 4. Pernicious anemia. Gradual relapse during 5½-month period of Congo red therapy.

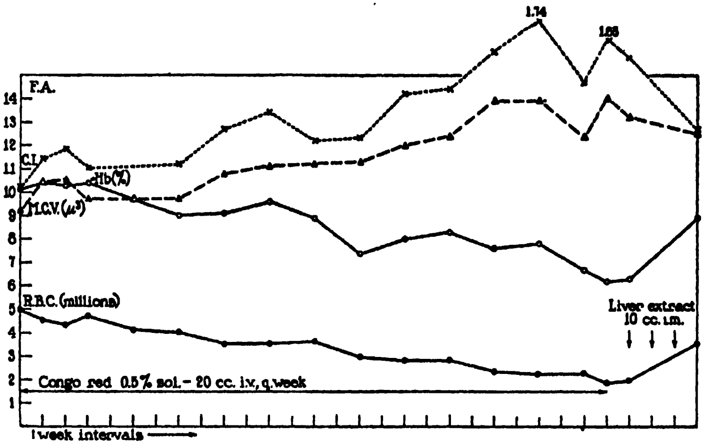

Chart V.—Case 5. Pernicious anemia. Gradual relapse during 6-month period of Congo red therapy.

Case 5.—F. A., male aged 59 (Chart V). Onset of weakness, sore tongue, and yellow pallor in 1929. Hgb. was 35%. On oral liver extract marked symptomatic improvement and Hgb. rose to 95%. Five years later, relapse because of inadequate therapy. April, 1935, admission to the hospital. *Physical examination:* Emaciation. Lemon-yellow pallor. Marginal papillary atrophy of tongue. Neurologic examination normal. *Blood:* R.B.C. 1,220,000; Hgb. 33%; C.I. 1.32; W.B.C. 3150; plasma bilirubin 2.65 mg. %; *Gastric analysis:* No free HCl even after histamine. *Course:* Reticulocyte peak of 25%, 5 days after intramuscular liver extract; R.B.C. and Hgb. returned to normal and patient was maintained in good health on 10 cc. of liver extract intramuscularly every 2 weeks. On September 23, 1935, R.B.C. 4,970,000; Hgb. 101%; M.C.V. 92; C.I. 1.02. Therapy changed to 20 cc. of Congo red solution once a week. At the end of 25 weeks R.B.C. 1,990,000 and Hgb. 63% with M.C.V. 132 and C.I. 1.58; moderate weakness his only complaint. Intramuscular liver-extract therapy resumed with rapid return of blood levels to normal.

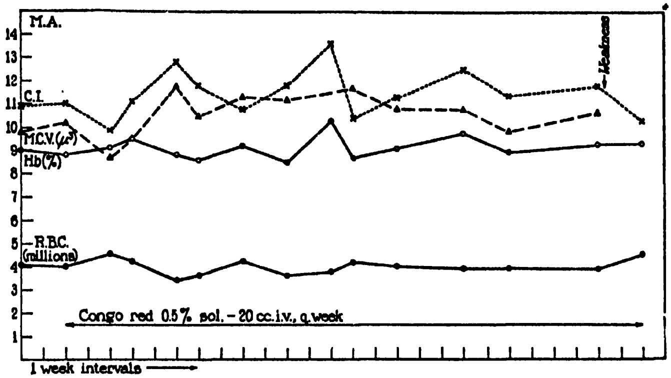

Chart VI.—Case 6. Pernicious anemia. Course of blood count during 6-month period of Congo red therapy. In contrast with Cases 1 to 5 this patient showed no essential change in blood picture.

Case 6.—M. A., female aged 37 (Chart VI). Three-year history of weakness, pallor, sore tongue and anemia. Temporary remission on liver extract. Discontinued therapy and admitted in relapse in February, 1935. *Physical examination:* Lemon-yellow pallor. Extreme papillary atrophy of tongue. Palpable spleen. Sluggish tendon reflexes. *Blood:* R.B.C. 1,115,000; Hgb. 35%; M.C.V. 134; C.I. 1.51; W.B.C. 3500. *Course:* Reticulocytes rose to 21% following intramuscular liver extract; R.B.C. and Hgb. returned to normal. Complete remission maintained with 10 cc. liver extract intramuscularly every 2 weeks. On October 10, 1936, R.B.C. 4,010,000; Hgb. 88%; M.C.V. 102; and C.I. 1.10. Therapy changed to 20 cc. Congo red solution intravenously once a week. After 25 weeks, no definite symptoms of relapse and no appreciable change in blood levels. Therapy changed back to liver extract.

Case 7.—F. G., Italian male aged 54 (Chart VII). Admitted to hospital September 3, 1935. History of weakness, loss of weight, and dizziness for 2½ years. Temporary improvement on oral liver. Symptoms recurred when he stopped liver therapy. Developed anorexia, abdominal discomfort, and paresthesias in extremities. *Physical examination:* Moderate pallor. Marginal papillary atrophy of tongue. Slight diminution of vibratory sense in feet. *Blood:* R.B.C. 2,490,000; Hgb. 76%; M.C.V. 133; C.I. 1.52; W.B.C. 4400. *Gastric analysis:* No free HCl, even after histamine. *Course:* Patient was placed upon meat-free diet for several weeks; weakness and indigestion increased; tongue became sore. Reticulocytes stabilized below 2%. Congo red solution (20 cc.) given daily for 6 days, followed by 40 cc. daily for 5 days. No rise in reticulocytes in 2 weeks. Patient then given single injection of 5 cc. liver extract (the amount derived from 25 gm. of whole liver) intramuscularly. Reticulocytes reached peak of 9% on 6th day with marked subjective improvement. Complete blood remission followed further liver-extract therapy, and for the past year the patient has remained symptom-free with normal blood levels on 10 cc. of liver extract intramuscularly every 2 weeks.

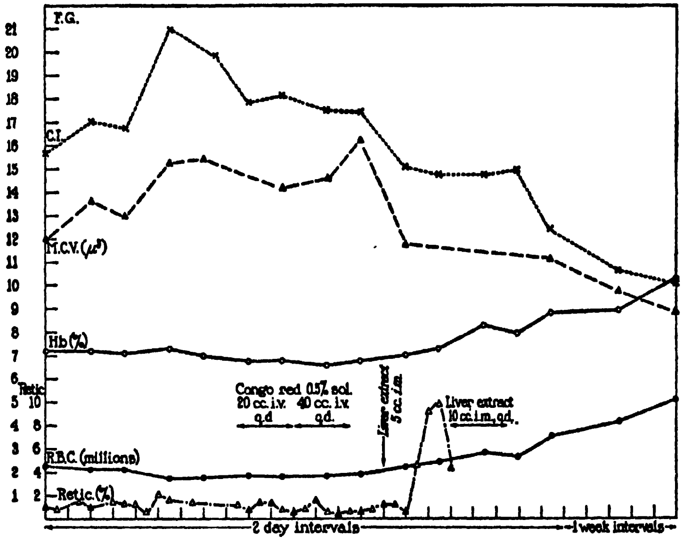

Chart VII.—Case 7. Pernicious anemia in relapse. Failure of daily injections of Congo red to modify the blood picture. Subsequent response to liver extract.

Case 8.—R. D., female aged 50, had lived in China 21 years. During the last 7 years of her stay she suffered from repeated attacks of diarrhea associated with soreness of tongue and mouth. Temporary relief of symptoms on diet of liver and sour milk. Later recurrence; oral liver extract failed to give relief. Severe anemia developed. Patient admitted to the hospital in 1931. *Physical examination:* Marked emaciation and pallor. Papillary atrophy of tongue. Palpable spleen. *Blood:* R.B.C. 820,000; Hgb. 22%; M.C.V. 131; C.I. 1.34; icteric index 20. *Gastric analysis:* Free HCl present only after histamine injection. *Course:* With intensive parenteral liver-extract therapy, diarrhea ceased, soreness of tongue cleared, and blood levels returned to normal. For the next 4 years she remained symptom-free on sprue diet augmented by an intramuscular injection of 10 cc. of liver extract every 2 to 3 weeks. If she tried to increase the interval between liver-extract injections to 4 weeks or longer, she invariably developed gastro-intestinal symptoms which were in turn rapidly alleviated by a single injection of liver extract. In December, 1935, therapy was changed from an intramuscular injection of liver extract once every 3 weeks to weekly intravenous injections of 20 cc. of Congo red solution. The diet was kept constant. Within 4 weeks of the last liver extract injection nausea and diarrhea set in. These symptoms increased in severity; at the end of 6 weeks the Congo red injections were abandoned and an injection of 10 cc. of liver extract was given intramuscularly. Within 36 hours of this liver extract injection, all symptoms had vanished.

CASE 9.—B. M., female aged 37, had resided for 12 years in Cuba. For 7 years recurrent attacks of diarrhea, nausea, vomiting, and abdominal cramps. Two years after onset of diarrhea sore mouth and anemia developed. Temporary improvement on sprue diet and oral liver extract; later symptoms recurred and failed to respond to oral therapy. Admission to hospital February, 1935. *Physical examination:* Marked pallor. Papillary atrophy of tongue with aphthous ulceration of buccal mucosa. Abdominal distention. *Blood:* R.B.C. 2,000,000; Hgb. 66%; M.C.V. 135; C.I. 1.65. *Gastric analysis:* Free HCl present after alcohol test meal. *Course:* On intramuscular and intravenous liver extract symptoms ceased and blood levels returned to normal. After discharge from the hospital patient was symptom-free for nearly a year on a sprue diet with a single intramuscular injection of 10 cc. of liver extract every 2 weeks. She then lapsed treatment and soon developed severe diarrhea and soreness of the tongue. She was readmitted to the hospital and given daily intravenous injections of 20 cc. of Congo red solution for 1 week. During this period diarrhea continued and a crop of aphthous ulcers appeared in the mouth. She was then given daily intramuscular injections of liver extract for 5 days. Soreness of the tongue disappeared after the first injection and the aphthous ulcers healed rapidly. Diarrhea ceased after the third injection of liver extract. The patient has since been maintained in excellent health on injections of liver extract once or twice a week.

Comment. There is obviously a marked discrepancy between the results that we obtained with Congo red and those reported by Massa and Zolezzi. Recently Lendvai,[4] in Budapest, has published a series of 12 cases of pernicious anemia treated with intravenous Congo red.

All of his patients were in severe relapse with erythrocyte counts varying from 800,000 to 2,200,000 red blood cells per c.mm. and hemoglobin levels of 28 to 64%. They received from 8 to 20 injections of 10 cc. of a 0.5% solution of Congo red over periods of 10 days to 4 weeks. Reticulocytes were followed in all cases and 4 cases showed slight reticulocyte rises of 4 to 8%; there was no increase in the reticulocytes in the other 6 cases. In no case did the red blood cell count or the hemoglobin rise above the pretreatment levels. Subsequent treatment with liver effected a complete blood remission in every case. Bone marrow was aspirated at intervals during the period of observation; the smears from the marrow presented a megaloblastic picture before and after Congo red therapy but a normoblastic picture after liver-extract therapy. Lendvai regards the slight reticulocytosis noted in certain of his cases as non-specific in type and concludes that Congo red exerts no therapeutic effect in pernicious anemia. The results of Lendvai are wholly in accord with those that we obtained in the treatment of pernicious anemia with Congo red.

Since the two cases of Mermod and Dock were changed to liver therapy as soon as reticulocyte rises had been produced with Congo red, there is no evidence that these reticulocyte crises differed in any way from the non-specific responses noted by Lendvai. Certainly there is no justification for assuming a specific therapeutic effect of Congo red in these 2 cases.

Thus the contention of Massa and Zolezzi that Congo red may bring about a complete blood remission in pernicious anemia lacks definite confirmation to date. It is quite impossible to reconcile the results reported by the Italian authors with our observations or those of Lendvai. One can only suggest: 1, that the Italian cases did not have true pernicious anemia, or 2, that they may have been individuals with "incomplete pernicious anemia." Goldhamer,[8] has demonstrated the presence of a small amount of intrinsic factor in the gastric juice of patients with pernicious anemia. It is conceivable that the gastric secretion of Massa and Zolezzi's responding cases contained a fair amount of intrinsic factor; that these patients had had diets poor in extrinsic factor prior to their admission to the hospital; and that they had received far better diets in the hospital. If such were the case, they might then have been able to manufacture through the combination of extrinsic and intrinsic factors in their gastro-intestinal tracts sufficient "liver extract" to bring about blood remissions. In other words, the remissions may have been dietary in origin and not due to the Congo red.

Finally, let us assume for the moment on the basis of Massa and Zolezzi's observations that Congo red might exert some therapeutic effect in pernicious anemia. Even so, Congo red could not be compared with the active principle of liver extract, for Dakin and West[2] have shown that 80 mg. of their purified fraction is sufficient to bring about a maximal reticulocyte response and an increase in the blood levels of patients with pernicious anemia. If Congo red even approached this active principle in potency, then our 2 cases in relapse and the 10 cases of Lendvai, which received 400 to 2100 mg. of the dye over relatively short periods, should certainly have shown striking improvement in both the clinical and the hematologic picture.

Conclusion. Congo red is totally ineffective in the treatment of patients with pernicious anemia or sprue. The dye may occasionally produce slight non-specific reticulocyte rises in cases of pernicious anemia.

REFERENCES

(1.) Barker, H.: J. Clin. Invest. (Proc.), **15**, 456, 1936. (2.) Dakin, H. D., and West, R.: J. Biol. Chem., **109**, 489, 1935. (3.) Goldhamer, S. M.: Am. J. Med. Sci., **191**, 405, 1936. (4.) Lendvai, J.: (*a*) Gyógyászat, **76**, 145, 1936; (*b*) Klin. Wchnschr., **15**, 1034, 1936. (5.) Mermod, C., and Dock, W.: Science, **82**, 155, 1935. (6.) Massa, M., and Zolezzi, G.: (*a*) Gior. di clin. med., **14**, 975, 1933: (*b*) Klin. Wchnschr., **14**, 235, 1935.

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IMMUNIZATION OF GUINEA PIGS WITH A MODIFIED STRAIN OF LYMPHOCYTIC CHORIOMENINGITIS VIRUS

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Two strains of lymphocytic choriomeningitis virus obtained in 1935 from naturally infected stock mice have been used for experimental work in this laboratory. These strains were highly virulent for guinea pigs when isolated. The present paper deals with the effect on the virus of serial passage through guinea pigs and mice and the use of a modified strain as an immunizing agent for guinea pigs.

The Strains Employed

Strain A, which was used in most of the experiments reported in previous papers (1, 2), showed no marked change in the course of 8 serial passages through guinea pigs by subcutaneous injection[1] or 10 serial transfers in virus-free mice by intracerebral inoculation. The mouse passages were discontinued after the 10th transfer, but the guinea pig passage strain has been maintained.

Strain B was obtained from two guinea pigs, both of which developed severe choriomeningitis after intracerebral inoculation with the brain of a mouse and were killed when moribund on the 12th or 13th day. Virus from their brains has thus far been passed in series through 14 guinea pigs by subcutaneous injection and in another series through 34 virus-free mice by intracerebral inoculation. Brain suspensions were used for subinoculation in guinea pigs and mice.

Effect on Strain B of Serial Passage through Guinea Pigs

The transfers through guinea pigs have maintained the high virulence of strain B for this species. Subcutaneous or intracerebral injections are usually fatal, even when very small amounts of virus are given, such as 1 to 10 minimal infective doses (M.I.D.), and the guinea pigs that do not die develop a very severe disease with marked emaciation and slow recovery.

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[1] Subcutaneous inoculations were made into the metatarsal pads.

All mice injected intracerebrally with this strain become sick, but many of them survive and fail to show the striking convulsions which characterize the disease induced by intracerebral injection with the mouse passage strain. Intravenous or intraperitoneal injection causes illness in the majority of the mice, but the rate of mortality is considerably lower than after intracerebral inoculation. Subcutaneous injection and intranasal instillation produce no symptoms and yet render mice resistant to intracerebral inoculation. The virus can be demonstrated in the blood of infected mice and occasionally persists in it for more than a month after recovery.

Effect on Strain B of Serial Passage through Mice

The results of intracerebral tests show that mouse passage has increased the virulence of strain B for mice. Subclinical infections occasionally occurred in the early passages even when large amounts of virus were inoculated, but injections with more than 10 M.I.D. of virus from the later passages caused typical convulsions and death on the 6th or 7th day in nearly 100 per cent of the mice. Doses near the borderline of infectivity, however, occasionally produced immunity without apparent symptoms. Intravenous, intraperitoneal, subcutaneous, or intranasal inoculations of virus from the later passages have failed to cause any definite signs of illness, but have immunized against intracerebral injection with virus. The blood of three mice injected intracerebrally was infectious when symptoms were manifest, but the virus was not demonstrated in the blood in repeated tests made 1 month after inoculation.

The pathogenicity of the mouse passage strain for guinea pigs has greatly decreased. It is not known exactly when this loss of virulence occurred; brain suspension from the 8th serial transfer was avirulent for guinea pigs. Intracerebral injections of this modified virus are followed by fever lasting several days and no other sign of disease. Some guinea pigs do not gain weight during the febrile period as rapidly as uninjected controls, but to an uninformed observer they do not appear ill. None of the symptoms that characterize the infection with the original virus or the guinea pig passage strain, such as labored respiration, drowsiness, salivation, vomiting, conjunctivitis, and emaciation, have been noted, and all of the 53 guinea pigs injected either intracerebrally or subcutaneously survived. Subcutaneous inoculations cause fever in the majority of the cases, but it is usually lower and of shorter duration than that induced by intracerebral injection. The amount of virus inoculated either intracerebrally or subcutaneously did not markedly influence the reaction of the animals. The blood contained virus during the febrile reaction on the 7th day after injection in two guinea pigs tested.

The virulence of the modified strain for guinea pigs was not restored by a single guinea pig passage, no matter whether blood or brain was used for subinoculation. Intracerebral or subcutaneous injection with a mixture of mouse brain containing modified virus and guinea pig brain in which the virulent strain had been inactivated by heating at 70°C. for ½ hour, likewise failed to increase the virulence of the modified strain. Nor has it been possible to modify the virulent guinea pig passage strain by one or two intracerebral passages through mice. This fact indicates that the modification of the mouse strain probably did not take place during the first two serial transfers in mice.

In the following section of the paper the strain B passed through mice and attenuated for guinea pigs will be called "mouse virus," while the strains A and B passed through guinea pigs and highly virulent for this species will be referred to as "guinea pig virus." For immunity tests with guinea pigs, strains A and B were often mixed to insure a high degree of virulence in the inoculum.

Immunization of Guinea Pigs with Mouse Virus

A single intracerebral or subcutaneous injection with mouse virus was sufficient to induce a high degree of resistance to intracerebral or subcutaneous inoculation with a large amount of guinea pig virus, such as 0.1 or 0.5 cc. of a 10 per cent guinea pig brain suspension, given 3 weeks later. The development of fever in some guinea pigs following subcutaneous inoculation apparently had no relation to the degree of immunity produced.

The immunity arose rapidly as Table I shows. Subcutaneous injection of mouse virus into the left hind pad followed immediately by a similar injection of guinea pig virus into the right hind pad failed to prevent the disease induced by the latter strain, but there is evidence that its course was modified in two animals (Nos. 4 and 5). On the 4th day a degree of resistance was apparent, and on the 8th or 10th day, 6 of the 9 guinea pigs tested were completely resistant,

TABLE I

Immunity of Guinea Pigs in Relation to Circulating Antivirus

Guinea pig No.	Route of inoculation with mouse virus	Test for antivirus in serum drawn 2–6 hrs. before test of immunity	Test of immunity by injection with guinea pig virus				
			Time after inoculation with mouse virus	Route of inoculation	Reaction	Controls	
						No.	Reaction
1	sc		1 min.	sc	D 11	17	D 14
2					D 13	18	D 15
3					D 27	19	D 9
4					Mild disease, rapid recovery	20	D 11
5					Severe disease, slow recovery	21	D 14
6	sc	–	4 days	sc	Fever	22	D 12
7		–			Mild disease, rapid recovery	23	D 12
8	sc	–	8 days	ic	Slight fever	24–27	D 11–15
9		–			None		
10		–			None	28–30	Very severe disease, slow recovery
11	sc	–	10 days	ic	Slight fever	31	D 11
12		+			Slight fever		
13		–			None		
14	ic	+			None	32	D 12
15		–			None		
16		–			None		

sc = subcutaneously.
ic = intracerebrally.
D 11 = died on 11th day.
– = no antivirus detected.
+ = antivirus probably present.

while the remainder developed a transient fever. Other guinea pigs not recorded in the table, which were tested for immunity by intracerebral inoculation on the 18th, 21st, or 31st day after subcutaneous or intracerebral injection with mouse virus, were all completely resistant.

The Relation of Circulating Antivirus to the Immunity of Guinea Pigs.—The failure to detect circulating antivirus in mice possessing a high degree of immunity to choriomeningitis was reported in a previous communication (1). Numerous other sera from mice immunized or hyperimmunized by a variety of methods have since been tested but none of them had definite neutralizing power. If antivirus was present, its concentration was too low to account for the high degree of resistance shown by the mice. Extracts of the brains and livers of mice that had become resistant to intracerebral injection by subcutaneous inoculation with mouse virus likewise failed to give any evidence of an antiviral substance. The sera of immune guinea pigs, on the other hand, usually contained antivirus when tested a few weeks after recovery.

Because of this apparent difference in mechanism between the immunity of mice and that of guinea pigs, an attempt was made to determine whether the immunity of the latter was due solely to circulating antivirus or, as in mice, to another factor as yet unknown but closely associated with the tissues affected by the virus.

A number of guinea pigs injected subcutaneously or intracerebrally with mouse virus were bled by heart puncture under deep ether anesthesia a few hours before they were tested for immunity as indicated in Table I. The sera obtained were avirulent for mice, except that of guinea pig 11 which produced the disease in one of the two mice inoculated. Neutralization tests were performed with the sera according to the method already described (1).

The results of the tests recorded in Table I indicate that the high degree of immunity present on the 8th and the 10th day after injection was not associated with demonstrable antivirus in the majority of the animals. Other guinea pig sera drawn 1 month after injection with mouse virus had definite neutralizing properties.

DISCUSSION

Strains of choriomeningitis virus isolated in earlier experiments from mice of the infected stock (2) often differed in virulence for guinea pigs although similar in every other respect. Some caused merely a febrile reaction in the majority of the animals, while others induced a very severe, fatal disease. However, not one of the strains of low virulence was quite consistent in its effect, a fatal disease sometimes resulting with those that were relatively non-pathogenic. The variations in severity of the disease were thought to be mainly due to differences in the susceptibility of guinea pigs, and this assumption was supported by the fact that the virus obtained from the same mouse at different times was differently pathogenic for guinea pigs (1, Table III). However, the rapid and striking change of strain B brought about by a few serial passages through mice now suggests that variations of the virus may be in part responsible for those differences. It has been shown that guinea pigs from the same stock as those used before react uniformly both to the guinea pig passage strain, which invariably causes very severe choriomeningitis, fatal in 80 to 90 per cent of the cases, and to the modified mouse strain, which has failed to produce symptoms in any of the 53 guinea pigs injected.

The modified virus is a very good immunizing agent for guinea pigs, since it is harmless to the animals and produces a solid immunity after a single injection.

The immunity to choriomeningitis of guinea pigs and mice differs in that immune guinea pigs as a rule develop circulating antivirus, whereas the sera from immune mice thus far tested have had no definite neutralizing properties. The comparatively late appearance of the antivirus in the guinea pig indicates, however, that it does not solely account for their immunity, which seems to be closely associated with the tissues affected by the virus. Similar observations have been reported for other viruses, for example, foot-and-mouth disease (3) and poliomyelitis (4–6). The possibility of infecting immune animal tissues *in vitro* with a variety of viruses, *e.g.*, virus III (7), vaccinia (8), herpes simplex (9), the salivary gland virus of guinea pigs (10), and pseudorabies (11), does not necessarily weigh against this conception of the mechanism of antiviral immunity. The washing and soaking of the tissue fragments, which usually preceded the infection in such experiments, may have removed an antiviral substance that was fixed in the tissues or rendered it ineffective by dilution. The evidence thus far obtained with tissue extracts from immune mice does not favor the assumption that such a substance is present, but further tests are necessary to disprove its existence. It is also possible that the removal of the tissue from its physiological environment and its maintenance under extremely unfavorable conditions alter the permeability to viruses of the cellular membrane. This permeability may provide the basis of susceptibility and immunity.

SUMMARY

A strain of choriomeningitis virus which was highly virulent for guinea pigs, as isolated from a naturally infected white mouse, has been markedly attenuated for guinea pigs by serial intracerebral passage through white mice. The change of virulence occurred before the 8th serial passage. The modified virus as a rule produces fever in guinea pigs but no other symptoms, and the infection is followed by a very solid immunity. Parallel passages of the same strain through guinea pigs have maintained its high virulence for this species but slightly reduced its pathogenicity for mice. These observations indicate that the differences in virulence for guinea pigs noted before (1, 2) with different strains of choriomeningitis virus obtained from infected stock mice may be due not only to differences in the susceptibility of guinea pigs but also to variations in the virulence of the virus.

A marked degree of resistance was demonstrable in several guinea pigs on the 4th, 8th, and 10th day after injection with modified virus, when antivirus could not yet be detected in the serum. Circulating antivirus appears therefore to play a secondary part in their immunity, which seems to be closely associated with the tissues as in mice.

BIBLIOGRAPHY

1. Traub, E., *J. Exp. Med.*, 1936, **63**, 847.
2. Traub, E., *J. Exp. Med.*, 1936, **63**, 533.
3. Waldmann, O., and Trautwein, K., *Centr. Bakt.*, *1. Abt.*, *Orig.*, 1923, **90**, 448.
4. Schultz, E. W., and Gebhardt, L. P., *California and West. Med.*, 1935, **43**, 111.
5. Jungeblut, C. W., *J. Infect. Dis.*, 1936, **58**, 150.

6. Sabin, A. B., and Olitsky, P. K., *J. Exp. Med.*, 1936, **64**, 739.
7. Andrewes, C. H., *Brit. J. Exp. Path.*, 1929, **10**, 273.
8. Rivers, T. M., Haagen, E., and Muckenfuss, R. S., *J. Exp. Med.*, 1929, **50**, 665, 673.
9. Andrewes, C. H., *J. Path. and Bact.*, 1930, **33**, 301.
10. Andrewes, C. H., *Brit. J. Exp. Path.*, 1930, **11**, 23.
11. Traub, E., *J. Exp. Med.*, 1935, **61**, 833.

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SEROLOGICAL STUDIES OF PLANT VIRUSES

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The serological approach to the problem of plant-virus classification has proved a fruitful field of study in recent years. A number of the plant viruses may be identified and classed in groups of closely related strains by means of the precipitin test (2, 3). A modified precipitin method has been described recently, whereby it is possible to identify viruses rapidly in the field with very little equipment (7). A considerable number of plant viruses, however, have regularly failed to give precipitin reactions (6). Part I of this paper is designed to report the progress made up to the present in classifying plant viruses by serological means and the present limitations of the method. A second section is devoted to studies on the techniques of plant-virus serology.

Part I. Serological Evidence in Plant-Virus Classification and the Limitations of the Method

The groups of serologically related viruses, as defined in a previous paper (3), have been subjected to further study with the following results.

Potato Aucuba-mosaic Viruses. Juices containing the virus of aucuba mosaic of potato (not to be confused with aucuba mosaic of tomato, which is a strain of tobacco mosaic) have been shown to give a specific precipitin test. Until recently, however, no strains of this virus, other than the typical strain from potato, have been encountered. A few months ago, T. P. Dykstra requested a serological determination of the Canada-streak virus of potato. Juices containing this virus reacted with serum prepared against the aucuba-mosaic virus and antiserum for Canada-streak precipitated aucuba-mosaic juices. Infection tests of the 2 viruses on a number of hosts indicated that they were very similar; hence, it is concluded that Canada streak is a strain of potato-aucuba mosaic.

Etch Viruses. According to the precipitin test, etch and severe etch are strains of the same virus group. The rare cross reactions with tobacco mosaic, mentioned in an earlier paper (3), have not been obtained in many repetitions of the test, and, hence, were probably due to a contamination of the cross-reacting materials. Blakeslee's Z-mosaic of Datura regularly interacts with the etch-virus sera and, accordingly, is looked upon as a strain of the etch group.

Cucumber-mosaic Viruses. The close serological relationship between cucumber mosaic and the veinbanding virus of potato (3) has been confirmed in many experiments involving the viruses of these 2 diseases. A number of the strains of cucumber-mosaic virus, isolated experimentally by Price (13), when tested by the field precipitin technique, proved to be serologically indistinguishable from cucumber-mosaic and veinbanding viruses. On the other hand, juices containing the viruses of celery mosaic and lily mosaic, both considered by Price to be strains of cucumber mosaic (14, 15), each failed to react with sera for any of the cucumber-mosaic group. The reciprocal tests, *i.e.*, preparation of sera for celery mosaic and lily mosaic and testing these sera against their proper viruses and against cucumber-mosaic juices, have not been performed. Doolittle's cucumber mosaic (characterized by more severe distortion on tobacco than Price's cucumber mosaic) also failed to react with sera for Price's cucumber mosaic or veinbanding.

Of 2 Canadian tobacco viruses received in December, 1936, from G. H. Berkeley, precipitin tests showed one to be an exceptionally mild strain of potato-veinbanding virus, the other a mixture of the veinbanding virus and tobacco ring spot. These results were confirmed by Berkeley's infection tests. An authentic sample of the Y-virus of European workers, kindly provided by T. P. Dykstra, proved to be serologically indistinguishable from the other viruses of the cucumber-mosaic group. Stipple streak of potato, when freed from its usual admixture of potato latent mosaic, likewise reacted as one of the veinbanding group. In potatoes in the field, latent mosaic is almost invariably present, and, accordingly, stipple streak may be looked upon as a type of rugose mosaic (veinbanding plus latent mosaic).

Tobacco-mosaic Viruses. Up to the present, plant-virus serology has been confined almost exclusively to the testing of juices of systemically infected leaves and succulent stems. It would be very desirable to have available data on the serological behavior of other virus-infected plant tissues, and in connection with the potato viruses and tuber-indexing a knowledge of the serological activity of virus-infected tubers will be necessary. The writer has had occasion to test for tobacco-mosaic virus antigens in (a) roots grown by White's tissue-culture methods (19) and in (b) leaves of plants in which virus is restricted to local lesions. These 2 types of test resulted as follows.

Tobacco-mosaic and tomato-aucuba-mosaic viruses were readily detectable by the precipitin test in the juices of macerated root tissues from tissue cultures containing these viruses. A sample of similar roots infected with latent mosaic of potato gave no reaction, but it was later found that in the many transfers undergone by these roots the virus infection had been outgrown and that the roots were no longer infective.

Tobacco-mosaic virus was propagated in *Nicotiana langsdorffi* Weinm., where it caused local necrotic lesions but no systemic infection under the conditions of these experiments. The infected leaves were macerated and the juice expressed and concentrated by precipitation with ammonium sulfate. The resultant product gave a strong specific precipitin reaction with tobacco-mosaic-immune serum.

These 2 types of experiment indicate that the heavy-weight protein, isolated by Stanley from systemically diseased tobacco and tomato plants, and responsible for the precipitin reaction, also occurs in considerable amount in local-lesion infections of this virus and in chlorophyll-free root-tissue cultures.

Serological Grouping of Reactive Viruses. Precipitin tests up to the present, in this laboratory and elsewhere, have provided evidence that the plant viruses that have given group-specific precipitin tests may be classified as follows:

a. *Tobacco-mosaic group:*

Field-type tobacco mosaic (Johnson's tobacco virus 1).
Holmes' attenuated and masked strains of tobacco mosaic.
Jensen's yellow and necrotic tobacco-mosaic isolates.
Aucuba mosaic of tomato.

Johnson's tobacco virus 6.
Certain Belgian necrotic diseases of tobacco (Manil, 11).
Petunia mosaic in Japan (Matsumoto, 12).

b. *Potato-latent-mosaic group:*

Potato mottle (ordinary latent mosaic or healthy potato virus).
European X-virus.
Johnson's potato ring spot.
Spot necrosis (1).
Attenuated spot necrosis (1).
Potato D-virus (16).
Hyoscyamus IV virus (9).
British Queen streak.
Masked latent mosaic (symptomless on tobacco, 8).

c. *Potato-veinbanding group:*

Typical veinbanding virus of potato.
European Y-virus.
Cucumber mosaic.
Price's yellow and green cucumber-mosaic isolates Nos. 2, 6, 8.
Valleau's Delphinium virus 10729.
Stipple streak of potato.
[Rugose mosaic of potato = a strain of veinbanding + a strain of latent mosaic].

d. *Potato-aucuba-mosaic group:*

Potato aucuba mosaic.
Canada streak of potato.

e. *Etch group:*

Etch.
Severe etch.
Blakeslee's Z-mosaic of Datura.

f. *Tobacco-ring-spot group:*

Wingard's tobacco ring spot.
Yellow tobacco ring spot.
Price's tobacco-ring-spot isolates: "green ring spot," "ring spot No. 2."

g. *Pea-mosaic group:*

Osborn's pea mosaic No. 2.
Osborn's pea mosaic No. 3.

h. *Mild mosaic of potato:*

Typical mild mosaic (reaction weak, often indefinite).

Verplancke (18) has reported positive precipitin tests with serum and extracts of mosaic beet plants. His serum was negative towards extracts of beets with yellows, of mosaic dahlia, of potato with mottle, crinkle mosaic, mild mosaic, streak, streak mosaic, and leaf roll, of mosaic pelargonium or of monstera with anthurium mosaic. Verplancke's experimental procedures differed from the customary serological methods in a number of respects, and his findings were not confirmed by Gratia and Manil (10). The beet-mosaic reaction, accordingly, requires further study before including it in the list of specific plant-virus reactions.

By the use of special techniques (5), certain of the strains of the tobacco-mosaic group may be differentiated from one another, and the same may be said for certain strains of the latent-potato-mosaic group. With ordinary precipitin techniques, however, the viruses included in any one group are indistinguishable from one another, but no virus from any given group cross-reacts with viruses from any other group. From the serological point of view, all of the viruses included in any group behave as strains of the same virus type. In general, this classification agrees with the results obtained by a number of workers using infection tests, physical and chemical properties of the viruses, or acquired immunity tests as criteria of relationship.

Nonreactive Viruses. Juices containing the viruses of the following diseases have been tested for precipitin reactions. The techniques used were similar to those that gave positive results with the viruses enumerated above. In no case, however, was a virus-specific precipitin reaction obtained.

[1]*Aster yellows.
Peach yellows.

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[1] Viruses marked with an asterisk were tested both by the standard precipitin technique and the field method. The other viruses were tested only by the standard method and might possibly yield specific reactions by the field method.

Potato witches' broom.
Potato leaf roll.
Potato mild mosaic, J-strain (Schultz).
Potato mild mosaic, V-strain (Schultz).
Potato mild interveinal mosaic (supermild mosaic).
Potato mild circular mottle.
*Potato yellow dwarf.
Potato calico mosaic.
Potato spindle tuber.
Potato crinkle mosaic (crinkle of Schultz and Folsom, not crinkle of Murphy and McKay, nor of Quanjer).
Pea mosaic No. 1 (Osborn).
Bean mosaic.
Sugar-cane mosaic.
Sugar-beet curly top. (?)
Sugar-beet mosaic.
*Tomato spotted wilt.
*Celery mosaic }
*Lily mosaic } See discussion above.
*Crucifer mosaic.

The viruses that have failed to give specific precipitin tests in nearly all cases differ from those that yield precipitins in most or all of the following respects:

Serologically active viruses	*Serologically inactive viruses*
a. Readily transmissible mechanically, often at dilutions of 1:1000 or higher.	Mechanical transmissibility poor or lacking.
b. Relatively stable *in vitro.*	Relatively unstable *in vitro;* usually destroyed by aging 2 days or less at room temperature.
c. Thermal inactivation point relatively high, usually stable at 55° C. or higher.	Thermal inactivation point usually below 55° C.
d. Readily systemic.	Sometimes very poorly systemic.

Several possible explanations may be suggested as reasons why some viruses yield juices that react serologically, while other viruses fail to do so. Thus, failure to demonstrate seric reactions with the viruses listed above may be due to insufficient virus antigen in the juice, to antigenic inactivity of the virus juice, to instability of the virus antigen *in vitro*, or in the experimental animals, or to a combination of these factors. It is unlikely, but not impossible, that failure of a virus juice to react might in some cases be due to the fact that the supposedly healthy host juice, used for absorbing the sera, contains a masked strain of the virus in question, incapable of causing recognizable symptoms on the plant but capable of precipitating the antibodies specific for the virus in question. Observations indicate that the explanation differs with different viruses. For example, using frozen juices in precipitin testing, tobacco ring spot gives a much stronger reaction with its proper serum than do the etch viruses. On the other hand, when these 2 viruses are tested by the field method, the etches are much more active than tobacco ring spot. According to results with the ultracentrifuge (17), the etches have much more heavy-weight virus protein than tobacco ring spot, and all available evidence indicates that it is the heavy-weight virus proteins that produce the virus precipitin reactions. It is, therefore, apparent that, in the case of the etch group, poor tests after freezing the juices are due to the instability of the virus-antigen, present in abundance in fresh juice; on the other hand, the weaker field test with tobacco ring spot appears to be due to the lower concentration of virus-antigen in juices containing this virus.

Moreover, the field method demonstrates precipitin reactions with a number of viruses that fail to react if the juices are frozen (cucumber-mosaic strains, etch strains). In these cases it is evident that a failure to obtain reactions with the frozen juices is due to instability of the virus-antigens and not to their being present in insufficient amount. In the case of tomato spotted wilt, no precipitin reaction was obtained, nor indeed was one expected, since this virus is inactivated by heating for 10 min. at the temperature of rabbit's blood; hence any virus inoculated into a rabbit probably would be destroyed long before antibody formation could occur. A few of the very slow-moving or poorly systemic strains of tobacco mosaic fail to give good precipitin tests. In such cases the concentration of virus antigen is evidently the important factor, since such viruses are poorly transmissible mechanically, although Jensen's tests show that they are highly resistant to heat and to ageing *in vitro*.

The correlation that appears to exist between seric activity and virus resistance suggests that the viruses themselves are directly responsible for the precipitin tests; however, the possibility that the reactions are due to specific by-products of virus activity is by no means excluded.

The Limitations of Plant-virus Serology. It follows from the data presented that the serological approach to the problem of plant-virus classification is limited by the fact that many viruses fail to produce specific precipitin reactions. At the 28th annual meeting of The American Phytopathological Society, the suggestion was advanced (6) that the list of viruses amenable to serological investigation might be lengthened by the development of more sensitive techniques. The development of the field method for virus testing, shortly afterwards, showed that such was the case, since it added to the list of serologically active diseases a number of etch and cucumber-mosaic strains. It is very possible that further developments in the preservation and concentration of viruses, or perhaps the use of coldblooded animals in serum preparation, may enlarge the field of virus serology.

Even with the limitations of the method as they now exist, however, the viruses that do yield specific reactions are those that present the most difficult problems in determination on account of their widespread occurrence and extensive host ranges. The poorly transmissible viruses are in general well characterized by their vector relationships, where these are known, and they do not present a problem in identification and classification so great as that encountered in the case of the serologically active viruses. From this point of view, even with its limitations, the serological method affords an aid in classification of those viruses with which such an aid is most needed.

Part II. Technique and Applications of the Field Method for Identifying Viruses

An account recently has been published of a method for identifying viruses in the field, which appears to represent a specific agglutination of the suspended plastids of freshly expressed virus-containing juices (7). Since the publication of this account, the field method has been subjected to further study with the following results.

Objectivity. In an endeavor to test the field method under conditions that would rule out any personal factor, a number of experiments were devised in which the viruses to be tested were unknown to the operator. A representative experiment will be described. Of 16 plants of Turkish tobacco, 10 had been inoculated with a strain of latent mosaic that, according to numerous observers, is completely symptomless on tobacco, although it causes a striking necrotic disease of pepper. At the time of the experiment, the 16 plants were indistinguishable. The plants were given to a colleague who rearranged them arbitrarily on a greenhouse bench and assigned arbitrary numbers to them. The 16 numbered plants were next turned over to an assistant who made inoculations from each plant to 2 pepper seedlings. They were then put in the hands of a second assistant who was given a supply of latent-mosaic serum and instructed to perform field-method precipitin tests on each plant. The results of the precipitin experiment, which were available at once, showed that 9 of the plants contained specific virus antigen. Two weeks later, when the pepper seedlings showed infection, it was found that every plant that had yielded a positive precipitin reaction also yielded inoculum infectious to the pepper seedlings, while the serologically inactive plants yielded no material infectious to pepper. One of the 10 inoculated tobacco plants evidently failed to become infected. This was shown by its negative precipitin reaction and by the negative pepper tests. The identity of the inoculated tobacco plants was not known to any of the persons involved in the experiment until after the experimental results were all recorded.

Analogous experiments, performed with a number of other viruses, have been consistent in showing that the method is accurate with unknown viruses in the hands of relatively untrained operators.

Laboratory Use of the Field Method. The suggestion has been made (7) that the advantages of the field method might warrant its use in the laboratory as the basic method for precipitin testing of viruses. The experience of the past few months has shown that such is the case, and, in the writer's hands, the field method has almost completely replaced the older method for routine precipitin testing of virus juices. The advantages are its rapidity (1 hour as compared with 2 days, for juice preparations by the older method) and its greater sensitivity towards most viruses. Tobacco-ring-spot virus alone proved to be more sensitive to the frozen-juice method, as recounted above. In the laboratory the field method may be somewhat improved, as regards sensitivity, by centrifuging the freshly expressed juices as a sole preliminary step to testing. This reduces the amount of suspended material in the juices and, hence, makes a small precipitation more readily apparent. It also serves to precipitate the larger suspended fragments of the juices and, hence, reduces disturbance of the reactions due to spontaneous settling in the juices.

Artifact Reactions. Occasionally, a plant juice will autoprecipitate when alone or in the presence of normal or heterologous serum. Such artifact precipitations do not lead to false conclusions as to the identity of viruses, because their presence in control tubes causes all reactions with an autoprecipitating juice to be excluded from the experiment. Artifacts of this type are no more frequent with the field method than with the frozen-juice method. They are particularly frequent in the juices of over-mature, yellowing plants. Such juices show strong oxidation, but attempts to eliminate the artifacts by the addition of reducing agents were unsuccessful, as, also, were attempts to remove them by change in pH or salt concentration of the juices. Often 20 to 30 per cent of precipitin tests must be discarded for this reason, and it is to be hoped that further investigation on the metabolism of dying plants will lead to a practical method for eliminating such autoprecipitations.

Preservation of Sera. Since refrigeration is impractical in the field, experiments were performed in order to test various methods of preservation of sera at room temperature. Such experiments showed that treatment of the sera with $HgCl_2$ at 1:4000 (addition of 1 part $HgCl_2$ at 1:400 to 9 parts of serum) provided sera that, after 35 days at room temperature, were still sparkling clear and showed no measurable loss in titer. Nearly the same results were obtained by adding 1 part of 5 per cent phenol to 9 parts of serum. Unpreserved sera, kept at room temperature for 35 days, in some cases lost all their precipitating power and were badly contaminated with bacteria.

Optimal Amounts of Serum and Virus. The relative proportions of serum and virus, as given in the original account of the field method, have continued to prove most satisfactory, except that the tobacco-ring-spot reaction was slightly improved by a change from 2 parts virus juice + 3 parts 1:9 serum to 4 parts virus juice + 1 part 1:3 serum.

Future Applications of the Field Method. The field method of testing for viruses is now restricted to the few laboratories where sera can be readily prepared. It has been shown, however, that the test is usable in the hands of untrained operators, that the sera may be preserved for long periods at room temperature, and hence may readily be shipped, and that the cost of preparing sera is relatively low if large animals are used. From these facts it follows that the use of the field method might be easily extended to a widespread application through the preparation and distribution of virus-immune sera by a central supply organization. The most important responsibility of such a supply source would be maintenance of virus stocks in a pure condition. Such a use of sera would make it possible to test geographically localized viruses without the dangers involved in shipping the viruses themselves, and would make the precipitin method accessible to investigators and teachers on a scale now impossible.

The agglutination of suspended plastids, as seen in the virus reactions, also is apparent if the juices of normal plants are tested against nonabsorbed antinormal-plant sera. From this fact it seems probable that the field method might prove to be a useful adjunct to or substitute for the present methods of classifying plants by blood reactions. It is possible that the field method might lack the precision of the techniques used by Mez and others in plant sero-systematics, but the ease and rapidity of performance warrant a testing of the field method in this connection. The field method as used with the plant viruses, coupled with a dialysis of all juices (to remove carbohydrates and other artifact-producing non-proteins), might serve as a desirable addition to the techniques of sero-diagnosis.

SUMMARY

Recent precipitin tests indicate that: Canada streak of potato is a strain of aucuba mosaic of potato; Blakeslee's Z-mosaic of Datura is a strain of the etch group; Price's cucumber-mosaic isolates are strains of the cucumber-mosaic group, although celery-mosaic, lily-mosaic, and Doolittle's cucumber-mosaic juices failed to react with sera for

Price's cucumber mosaic; the European Y-virus of potato is serologically indistinguishable from the American potato-veinbanding virus; potato stipple streak also is of the veinbanding group, and, since, in the field, it is customarily associated with latent mosaic, it is considered to be a type of rugose mosaic. Tobacco-mosaic virus, propagated in root-tissue cultures and in locally necrotic lesions, yields specific virus antigen. All of the viruses that have thus far proved serologically active are grouped according to their relationship reactions. A list is given of the viruses that have failed to give reactions. Possible explanations for this failure are discussed.

The field method of precipitin testing for viruses gave dependable results in the hands of unskilled workers to whom the identity of the virus materials was unknown. Data and suggestions are given regarding the use of the field method as a laboratory procedure, the elimination of artifact reactions, the preservation of sera, and the applications of the field method of precipitin testing.

LITERATURE CITED

1. Birkeland, J. M. Serological studies of plant viruses. Bot. Gaz. **95**: 419–436. 1934.
2. Chester, K. S. Serological evidence in the study of the relationships of certain plant viruses. (Abstract) Phytopath. **25**: 10. 1935.
3. ———. Serological evidence in plant-virus classification. Phytopath. **25**: 686–701. 1935.
4. ———. The antigenicity of the plant viruses. Phytopath. **25**: 702–714. 1935.
5. ———. Separation and analysis of virus strains by means of precipitin tests. Phytopath. **26**: 778–785. 1936.
6. ———. The limitations of plant virus serology. (Abstract) Phytopath. **27**: 124. 1937.
7. ———. A simple and rapid method for identifying plant viruses in the field. Phytopath. **27**: 722–727. 1937.
8. ———. A critique of plant serology. Part II. Quart. Rev. Biol. **12**: 165–190. 1937.
9. Gratia, A., and P. Manil. Différenciation sérologique des virus X et Y de la pomme de terre chez les plantes infectées ou porteuses de ces virus. Compt. Rend. Soc. Biol. [Paris] **117**: 490–492. 1934.
10. ———, and ———. De quelques échecs de la méthode sérologique appliquée aux virus des plantes. Compt. Rend. Soc. Biol. **118**: 379–381. 1935.

11. Manil, P. Note sur les nécroses foliaires du tabac, dans les cultures de la vallée de la Semois, en 1934. Bull. Inst. Agron. et Stat. Rech. Gembloux. **3**: 367–377. 1934.
12. Matsumoto, T. Differentiation of two Petunia mosaic diseases by means of serological, cytological, and inoculation experiments. Bot. and Zool. [Tokyo] **3**: 893–898. 1935. [In Japanese].
13. Price, W. C. Isolation and study of some yellow strains of cucumber mosaic. Phytopath. **24**: 743–761. 1934.
14. ———. Classification of southern celery-mosaic virus. Phytopath. **25**: 947–954. 1935.
15. ———. Classification of lily-mosaic virus. Phytopath. **27**: 561–569. 1937.
16. Spooner, E. T. C., and F. C. Bawden. Experiments on the serological reactions of the potato virus "X". Brit. Jour. Expt. Path. **16**: 218–230. 1935.
17. Stanley, W. M., and R. W. G. Wyckoff. The isolation of tobacco ring spot and other virus proteins by ultracentrifugation. Science (n.s.) **85**: 181–183. 1937.
18. Verplancke, G. Étude des propriétés des virus causant les maladies de dégénérescence de la betterave. Sucr. Belge **54**: 118–127, 142–151. 1934; 162–168. 1935.
19. White, P. R. Plant tissue cultures. Bot. Rev. **2**: 419–437. 1936.

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A COMPARISON OF NUTRIENT SALT SOLUTIONS FOR THE CULTIVATION OF EXCISED TOMATO ROOTS

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A study planned to determine if any of a number of salt solutions chosen from the literature of plant and animal nutrition may be superior for isolated tomato roots to that used in previous studies.

The nutrient solutions employed in all studies of excised roots to date have been modifications of Knop solution ('65), with considerable changes in the proportions and absolute concentrations of the constituent salts but without important changes in the particular salts used. Kotte ('22), Malyschev ('32), and Gautheret ('35) used Knop solution, Robbins ('22) the closely related Pfeffer solution. White's modification of Uspenski solution ('34) more closely resembles Pfeffer solution than it does the original formula of Uspenski and Uspenskaia ('25). Formulae for many other nutrient salt solutions have been published in the literature of plant nutrition. These range in complexity from the 3-salt solution of Shive ('15) to the 8-salt solution of Trelease and Trelease ('33) and the even more complex "A-Z" solution of Hoagland and Snyder ('33). The possibility that some solution other than those previously used might prove more satisfactory for excised roots led to the testing of a considerable number of such solutions.

For this purpose, formulae of 14 standard solutions representing a wide range of compositions were chosen from the plant nutrition literature. Three solutions commonly used in animal nutrition studies (McCollum et al., '28; Ringer, '86; Tyrode, '10) were added for comparison. The concentrations of all constituents of these solutions were recalculated so that the total molar concentration of each solution should equal that of the modified Uspenski solution used in all of the author's work ('34), which will hereafter be referred to as

TABLE 1

No.	Salt	Grams in 1 liter of a 0.01 M solution		Standard	Knop*	Pfeffer*	Schimper*	Tottingham*	H.W. & P.*	Crone*	Sachs*	Schreiner & Skinner*	Shive	Trelease & Trelease†	Mazé††	Marchal & Marchal	Beijerinck**	McCollum et al.	Ringer***	Tyrode***
		Anhydrous salt	Hydrate	Milliliters of a 0.01 M solution in 1 liter of nutrient = concentration in millimols multiplied by 100																
1	$CaCl_2$	1.11												66			25		15	20
2	$CaCO_3$	1.00													160					
3	$CaSO_4$	1.36	1.54							30	50					60				
4	$Ca(NO_3)_2$	1.64	2.36	60	130	115	100	85	115				35							
5	$Ca_3(PO_4)_2$	3.10								15	45									
6	$CaH_4(PO_4)_2$	2.34	2.98						25			5						35		
7	Ca-lactate	2.18	3.03															10		
8	KCl	0.74		90		30			65										20	20
9	KNO_3	1.01		80	55	50	40	30		175	110			110						
10	K_2SO_4	1.74										25				50				
11	K_2HPO_4	1.74					25							2	15		35	90		
12	KH_2PO_4	1.36		10	40	35		90					125	40	20					
13	K-citrate	3.06	3.24											1						
14	NaCl	0.58					70				20							35	205	110
15	$NaNO_3$	0.85										240			64					
16	$NaHCO_3$	0.84																	30	105
17	NaH_2PO_4	1.20																40		5
18	$MgCl_2$	0.95	2.03																	10
19	$MgSO_4$	1.20	2.46	30	45	40	35	65	65	40	45		110	40	10	70	25	50		
20	NH_4NO_3	0.80														20	185			
21	$(NH_4)_2SO_4$	1.32												10						
22	$(NH_4)_2HPO_4$	1.32														65				

23	Fe_2SO_4	1.52	2.64											1						
24	$Fe_2(SO_4)_3$	4.00		0.6	0.6	0.6	0.6	0.6	0.6		0.6	0.6	0.6		1.3	5.0	0.6		0.6	0.6
25	$Fe_3(PO_4)_2$	3.58								10										
26	Fe-citrate	2.45	3.00															10		
Total molar concentration				2.706	2.706	2.706	2.706	2.706	2.706	2.700	2.706	2.706	2.706	2.700	2.703	2.700	2.706	2.700	2.706	2.706
Bibliography number				'34	'65	'00	'90	'14	'07	'02	'60	'10	'15	'33	'19	'13	'93	'28	'86	'10

* Formulae taken from Tottingham ('14).

** Formula taken from Moore ('03).

*** Formulae taken from Bayliss ('24).

† Only the common salts of Trelease and Trelease's formula are included. The "accessory salts" have been omitted.

†† Only the common salts of formula are included. The "accessory salts" have been omitted.

"standard solution." This is essentially a Pfeffer solution with reduced phosphate content, having a salt concentration of 2.7 millimols. The salt proportions of all the plant-nutrient solutions were kept unchanged. Because of the use of NaCl in considerable concentration in Ringer ('86) and Tyrode ('10) solutions, as an osmotic agent, an exact proportionate transformation of these solutions to the molar concentration of the standard solution would have left the concentrations of cations other than Na much below the minimum requirements for plants. For this reason the NaCl concentration in these solutions was reduced by an amount 10 times as great as the other salts. This gave a final Na:K ratio approaching that of the few plant-nutrient solutions which regularly contain sodium (Schimper, '90; Sachs, '60; Schreiner and Skinner, '10; Mazé, '19). When the source and concentration of iron to be added was not specifically stated in the original reference, $Fe_2(SO_4)_3$ at the concentration regularly used in the standard solution, 0.006 millimol, was added. The salt concentrations of the solutions tested are given in table 1. Two per cent of sucrose and 0.01 per cent of yeast extract were included in all nutrients ('32). All roots used were derived from the standardized clone C ('34) and were selected for uniformity. All cultures were treated uniformly except for the variations in salt constituents under examination. Since it was not possible to test all solutions simultaneously, the results were calculated as percentages of the growth index in the standard solution kept as control in each series of cultures. Ten cultures were grown in each solution for one week and each series was repeated 3 times. All cultures were measured daily and the results averaged. The numerical indices given are thus the means of the results from 30 cultures grown at 3 different times.

The results of the tests are presented in table 2. While an unmodified Knop solution gave a higher growth index on the first day of the passage than did the control, and Shive solution gave higher indices on the 3rd, 4th, and 5th days, the *total* growth index for the entire passage was lower than that of the control in every other nutrient. In none of the solutions studied, with the exception of Shive solution, did the growth index approach that of the control closely enough to leave any doubt of the superiority of the latter as a nutrient for isolated tomato roots.

All of the first 9 solutions in table 2 gave fairly good results. It is probable that cultures *could* be maintained in them indefinitely, though at a low level of increment. These solutions were placed in group 1 in classifying the different solutions used. The last 8 solutions, constituting group 2, gave very poor results and the demarcation between the groups was quite sharp. At the end of one week each culture was transferred to a solution similar to that used in the first passage for one week and then all cultures in group 2 were returned to the standard solution to test if they had been injured in the experimental solutions. In the second passage the order of growth percentages within groups 1 and 2 differed somewhat. Shive solution dropped from second to fourth place. But all of the first 9 solutions again maintained growth 50 per cent of that in the control or better, while the last 8 solutions again gave little or no growth. In the third passage, carried out in the standard solution, only those cultures taken

TABLE 2

Solution	Growth expressed as percentages of the control										pH*
	Days							Total	Passage 2	Passage 3 (control)	
	1	2	3	4	5	6	7				
1. Standard	100	100	100	100	100	100	100	100	100	100	5.5
2. Shive	92	94	(116)	(109)	(109)	96	83	99	78	—	4.5
3. Tottingham	93	94	80	76	86	70	75	82	73	—	5.8
4. Knop	(121)	73	83	83	78	74	71	81	87	—	4.7
5. Schimper	74	87	85	79	75	66	83	78	76	—	4.8
6. Pfeffer	91	88	73	74	83	51	58	72	74	—	4.9
7. H. W. & P.	55	72	62	54	79	66	65	65	98	—	4.9
8. S. & S.	55	62	55	61	65	56	55	58	49	—	4.7
9. T. & T.	62	60	45	42	57	38	46	49	66	—	4.8
10. Crone	71	38	11	13	10	10	12	20	30	69	6.1
11. Mazé	86	30	8	8	1	1	1	14	0	0	7.2
12. Ringer	64	30	8	6	1	3	1	12	0	12	7.3
13. Beijerinck	55	25	11	9	3	1	1	12	0	16	6.4
14. McC., R. & B.	74	19	4	5	1	0	4	11	0	0	6.2
15. M. & M.	40	27	10	5	3	1	3	10	0	0	6.7
16. Tyrode	50	19	7	6	3	1	1	10	0	0	7.7
17. Sachs	43	25	6	4	3	1	1	9	0	9	6.1

* Determined with glass-electrode potentiometer.

from Crone solution ('02) recovered satisfactorily, although single roots transferred from the solutions of Beijerinck ('93), Ringer ('86), and Sachs ('60) showed some recovery. The solutions of Mazé ('19), Marchal and Marchal ('13), Sachs ('60), McCollum, Rask, and Becker ('28), Ringer ('86), and Tyrode ('10) were not merely inadequate to support growth but were actually injurious to isolated roots.

An exact analysis of all of the factors responsible for the differences in behavior of these various solutions will require a much more extensive study than has been possible in the time available for this work. An examination of the results presented in table 2 in the light of the formulae in table 1 shows, however, one striking correlation. All solutions in group 1 were relatively acid, all of them having reactions below pH 6.0. All solutions in group 2 were much less acid, having reactions regularly above pH 6.0. Reaction of the solution would thus appear to be of paramount importance for the growth of tomato roots.

Other factors are of less importance. McCollum solution, for example, falling in group 2 is characterized by a very high phosphate content, yet a high phosphate content is not universally objectionable, for Shive solution, standing second only to the standard solution in group 1, has 14 times the phosphate content of the latter. Of the 9 solutions falling in group 1, only 3 depart widely from the Knop solution type. These are the solution of Trelease and Trelease ('33) in which $CaCl_2$ replaces the $Ca(NO_3)_2$ of Knop solution (the addition of traces of K_2HPO_4 and $(NH_4)_2HPO_4$ are probably without much significance), that of Hartwell, Wheeler, and Pember ('07) in which $CaH_4(PO_4)_2$ replaces the KH_2PO_4 of Knop solution, and that of Schreiner and Skinner ('10). This last solution bears little resemblance to any of the other satisfactory solutions, and is anomalous in that it completely lacks magnesium in any form. It will be recalled, however, that earlier work of the author ('33) cast doubt on the importance of magnesium for the development of isolated chlorophyll-free plant tissues. This solution is clearly worthy of closer study, in spite of its low position (8th place) in the order of usable solutions. The marked superiority of the Knop type of solution in general is evident, and a study of the formulae in group 1 shows that within the Knop-Pfeffer-Shive type of formula a rather wide range of salt proportions is possible. As was shown elsewhere ('33), this is much more true of anion than of cation proportions.

Beijerinck solution ('93) has long been ranked as an excellent nutrient for algae, fungi, and sometimes for seed plants. Its ranking in 13th place in a series of 17, in the present study, was thus surprising. Since, however, this solution if unadjusted is relatively much less acid than are the satisfactory solutions and an acid reaction appears to be important for tomato roots, the effect of readjustment of the solution reaction was tested on Beijerinck solution and 3 other solutions. For this purpose 4 solutions, having unadjusted pH's of 4.5 (Shive), 5.5 (standard), 6.4 (Beijerinck), and 7.7 (Tyrode), were chosen and aliquots of each were adjusted to the hydrogen-ion concentrations characteristic of each other solution. Ten cultures were made at each reaction in each solution. The standard solution at pH = 5.5 served as control. The growth curves of these 16 sets of cultures over a single passage are shown in figure 1. At pH 4.5 Shive solution and Beijerinck solution were both superior to the standard solution at that pH, maintaining mean growth levels of approximately 6 mm. per culture per day. This is a fairly satisfactory growth index, though not equal to that of the unadjusted control. The standard solution and Tyrode solution gave about equal results, with indices around 4 mm. per culture per day. At pH 5.5 the standard solution was markedly superior to the others with a mean index of 9.3, while roots in Shive solution, Beijerinck solution, and Tyrode solution were poorer than they had been at pH = 4.5. At pH = 6.5 and 7.5, cultures grew very poorly in all four solutions.

The significance of these results will be clear if the unadjusted pH values of the four solutions (table 2) are remembered. The standard solution and Shive solution gave their best results at their unadjusted pH values, being injured by any adjustment tested. Beijerinck solution and Tyrode solution, which have reactions above pH 6.0 when unadjusted, were greatly improved by acidification. Beijerinck solution, which is of negligible value at its unadjusted pH of 6.5, became as effective as was Shive solution and almost as effective as the control when adjusted to Shive solution's normal pH of 4.5. Tyrode solution, which in some of its many modifications is extensively used in the cultivation of animal tissues, was entirely worthless for tomato roots if kept at its unadjusted pH, but if acidified to pH = 4.5 became fairly satisfactory, though the improvement was not sufficient to justify its extensive use with plant tissues. The initial failure of Beijerinck solution to give satisfactory results was thus not due to its constituents as such, but to the pH equilibrium brought about by the particular salt combination used. The degree of acidity of the nutrient, as was suggested by the results shown in table 2, was a matter

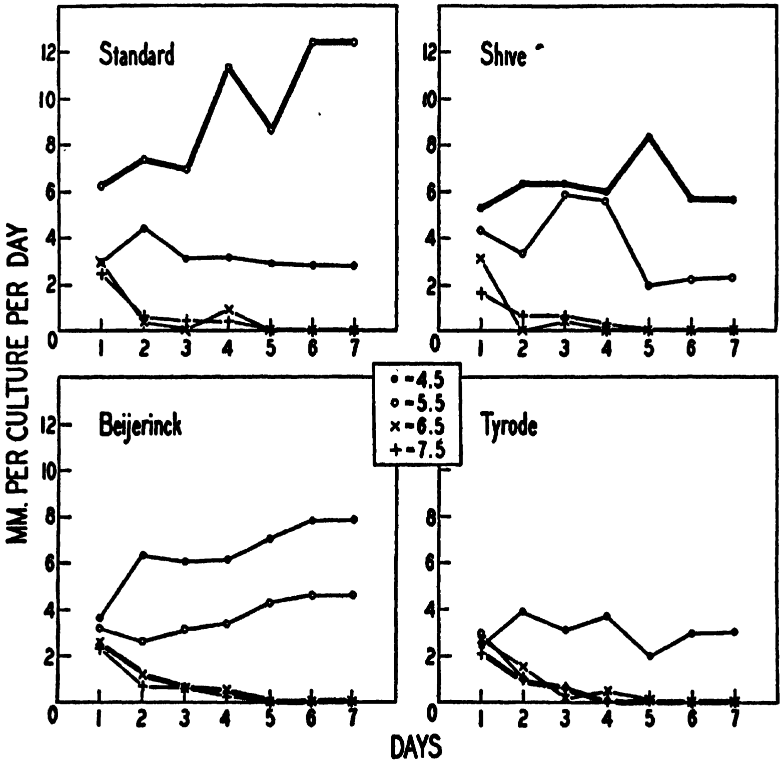

FIG. 1. Graphs showing daily increment rates of isolated tomato roots during one passage (7 days) in four different nutrient solutions, each at four different hydrogen-ion concentrations. Solid circles = pH 4.5, open circles = pH 5.5, crosses = pH 6.5, and plus signs = pH 7.5. The heavy line in each graph represents the increment rates at the unadjusted pH characteristic of the particular solution shown.

of prime importance. A distinctly acid medium is required by tomato roots. This is the more interesting in view of the neutral or alkaline reaction required by animal tissues.

Since the best results obtained in this series of tests were with the standard solution unadjusted, and since, when other solutions gave satisfactory results they did so at the same reaction or a more acid one than that normal for the standard solution, the author will continue to use this solution in future work.

SUMMARY

The relative efficiencies of 17 salt solutions as nutrients for isolated tomato roots were tested. The standard solution previously used in this laboratory gave the most satisfactory results, with Shive solution second. All satisfactory solutions had reactions below pH 6.0, all unsatisfactory ones had reactions above pH 6.0. Beijerinck and Tyrode solutions were greatly improved by acidification. An acid reaction thus appears to be necessary for satisfactory growth of isolated tomato roots.

LITERATURE CITED

Bayliss, W. M. 1924. Principles of general physiology. Longmans, Green and Co. London.

Beijerinck, M. W. 1893. Berichte über meine Kulturen neiderer Algen auf Nährgelatine. Centralbl. f. Bakt. **13**: 368–373.

Crone, G. 1902. Ergebnisse von Untersuchungen über die Wirkung der Phosphorsäure auf die höhere Pflanzen und eine neue Nährlösung. Sitzungsber. Niederrhein. Gesel. Nat. und Heilkunde, Bonn. **1902**: 167–173.

Gautheret, R. J. 1935. Recherches sur la culture des tissus végétaux: Essais de culture de quelques tissus méristematiques. Thése, Univ. de Paris. pp. 279.

Hartwell, B. L., H. J. Wheeler, and F. R. Pember. 1907. The effect of the addition of sodium to deficient amounts of potassium upon the growth of plants in both water and sand cultures. Ann. Rep. Rhode Island Agr. Exp. Sta. **20**: 299–357.

Hoagland, D. R., and W. C. Snyder. 1933. Nutrition of strawberry plant under controlled conditions. Proc. Am. Soc. Hort. Sci. **30**: 288–294.

Knop, W. 1865. Quantitative Untersuchungen über den Ernährungsprocess der Pflanzen. Landw. Versuchsst. **7**: 93–107.

Kotte, W. 1922. Kulturversuche mit isolierten Wurzelspitzen. Beitr. allg. Bot. **2**: 413–434.

Malyschev, N. 1932. Das Wachstum des isolierten Wurzelmeristems auf sterilen Nährboden. Biol. Ztrbl. **52**: 257–265.

Marchal, Él., and Ém. Marchal. (See Servettaz, C. 1913. Recherches expérimentales sur le développement et la nutrition des mousses en milieux stérilisés. Ann. Sci. Nat. Bot., Ser. 9, **17**: 111–224.)

Mazé, P. 1919. Recherche d'une solution purement minérale capable d'assurer l'évolution complète du mais cultivé à l'abri des microbes. Ann. Inst. Pasteur **33**: 139–173.

McCollum, E. V., O. S. Rask, and J. E. Becker. 1928. A study of the possible role of aluminum compounds in animal and plant physiology. J. Biol. Chem. **77**: 753–768.

Moore, G. T. 1903. Methods for growing pure cultures of algae. J. Appl. Microsc. **6**: 2309–2314.

Pfeffer, W. 1900. The physiology of plants. (Tr. by A. J. Ewart.) Oxford.

Ringer, S. 1886. Further experiments regarding the influence of lime, potassium and other salts on muscular tissue. J. Physiol. **7**: 291–308.

Robbins, W. J. 1922. Cultivation of excised root tips and stem tips under sterile conditions. Bot. Gaz. **73**: 376–390.

Sachs, J. 1860. Vegetationsversuche mit Ausschluss des Bodens über die Nährstoffe und sonstigen Ernährungsbedingungen von Mais, Bohnen, und anderen Pflanzen. Landw. Versuchsst. **2**: 219–268.

Schimper, A. F. W. 1890. Zur Frage der Assimilation der Mineralsalze durch die grüne Pflanze. Flora **73**: 207–261.

Schreiner, O., and J. J. Skinner. 1910. Ratio of phosphate, nitrate and potassium on absorption and growth. (sic.) Bot. Gaz. **50**: 1–30.

Shive, J. W. 1915. A study of physiological balance in nutrient media. Physiol. Researches **1**: 327–397.

Tottingham, W. E. 1914. A quantitative chemical and physiological study of nutrient solutions for plant cultures. Physiol. Researches **1**: 133–245.

Trelease, S., and H. M. Trelease. 1933. Physiologically balanced culture solutions with stable hydrogen-ion concentration. Science **78**: 438–439.

Tyrode, M. V. 1910. The mode of action of some purgative salts. Arch. Intern. Pharmacodyn. **20**: 205–223.

Uspenski, E. E., and W. J. Uspenskaia. 1925. Reinkultur und ungeschlechtliche Fortpflanzung des *Volvox minor* und *Volvox globator* in einer synthetischen Nährlösung. Ztschr. Bot. **17**: 273–308.

White, P. R. 1932. Influence of some environmental conditions on the growth of excised root tips of wheat seedlings in liquid media. Pl. Physiol. **7**: 613–628.

——. 1933. Concentrations of inorganic ions as related to growth of excised root tips of wheat seedlings. *Ibid.* **8**: 489–508.

——. 1934. Potentially unlimited growth of excised tomato root tips in a liquid medium. *Ibid.* **9**: 585–600.

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CHEMICAL STUDIES ON THE VIRUS OF TOBACCO MOSAIC

X. THE ACTIVITY AND YIELD OF VIRUS PROTEIN FROM PLANTS DISEASED FOR DIFFERENT PERIODS OF TIME

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The increase of virus in plants following inoculation with tobacco mosaic virus has been studied by Holmes (1) and by others (2–4). No detailed studies have been reported, however, of the rate of increase of tobacco mosaic virus protein in plants or of the physicochemical and biological properties of virus protein isolated from plants diseased for different periods of time. There are certain isolated observations which bear on this general problem. In previous work the increase of virus protein in one group of plants during a 4 week period was noted and estimated to consist of over a millionfold increase (5). It was found in other work that the virus activity of protein obtained from old plants was less than the activity of protein obtained from young plants (6). It is possible, however, that this difference might have been due to the more drastic purification procedure which was required for samples of the former. During the course of an ultracentrifugal analysis of various virus proteins, it was found, in three preliminary experiments, that the sedimentation constant of the virus protein varied irregularly with the age of the plants from which the protein was obtained (7). It is not known whether this variation was due to actual differences in the protein as it existed in the plants or to differences arising during the isolation and purification of the protein.

These observations indicated the necessity for a detailed study of the increase of virus protein in the plant and of the physicochemical and biological properties of virus protein in plants diseased for different periods of time. Such a study has been carried out with the ordinary strain of tobacco mosaic virus in Turkish tobacco plants and certain of the results are presented in this paper. The ultracentrifugal study on this same material is presented in the following paper (8).

EXPERIMENTAL

Preparation and Chemical Analysis of Plant Material—Representative sets, each consisting of from eight to sixteen Turkish tobacco plants about 3 inches high, were selected from a large group of plants growing in 6 inch pots and were placed in separate rows across a greenhouse bench. Half of these plants were inoculated on April 15, 1936, by rubbing two leaves on each plant twice with a bandage gauze pad which was kept moistened with a solution of 0.1 M phosphate buffer containing 10^{-3} gm. of tobacco mosaic virus protein per cc. One set of the uninoculated plants was cut and the remaining plants were used as normal controls. At weekly intervals up to and including 8 weeks after the time of inoculation, one set of diseased plants and one set of normal plants were cut and immediately placed in a room held at −8°. The sets of frozen plants were then put through a meat grinder and 4 per cent by weight of disodium phosphate in the form of a 50 per cent aqueous solution was added to the pulp. The pulp was allowed to thaw; the juice was pressed out and filtered through celite (Standard supercel) and its total nitrogen and protein nitrogen contents were determined as previously described (6). The pulp was then extracted with a volume of 0.1 M phosphate buffer at pH 7 equal to the volume of the juice or first extract and then dried to constant weight at 110°. The dry weight and total nitrogen content of the pulp were determined. In this first experiment the virus protein was isolated from the juices of the different sets of diseased plants by chemical methods which have been described (5). Although an attempt was made to work up each set in a comparable manner, the sets were processed at different times and after irregular periods of time following harvesting.

The analytical data for the nine sets of normal plants and the eight sets of diseased plants are presented in Table I. It may be seen from the green weights of the plants that the diseased plants were stunted and weighed considerably less than did the normal plants of the same age. However, with the exception of that of the plants of the 1st week after inoculation, the juice from the diseased plants contained considerably more total nitrogen and protein nitrogen than did the juice from normal plants of the same age. After 5 weeks the protein nitrogen in the juice of the diseased plants dropped off despite the fact that the total nitrogen content remained about constant. A similar situation prevailed in the case of the normal plants. The virus protein per cc. of juice from diseased plants obtained by chemical methods increased rapidly

TABLE I

Preparation by Chemical Means of Virus Protein from Turkish Tobacco Plants Harvested at Weekly Intervals Following Infection with Tobacco Mosaic Virus. Summary of Data for Normal and Diseased Plants

Time after inoculation	Green weight per plant	Yield of juice per plant	Yield of juice	Total N in juice	Protein N in juice	Dry weight of pulp, 110°	Total N per gm. dry pulp	Virus protein N yield per cc. juice	Protein N recovered as virus protein
				Diseased plants					
wks.	*gm.*	*cc.*	*per cent*	*mg. per cc.*	*mg. per cc.*	*gm. per plant*	*mg.*	*mg.*	*per cent*
1	67.4	53.7	79.8	1.36	0.38	2.51	40	0.02	4.4
2	120.3	107.0	88.0	(2.28)	0.71	7.40	27	0.16	23.2
3	164.5	126.8	77.0	1.08	0.71	7.88	26	0.24	33.4
4	206.2	145.8	70.5	1.07	0.71	11.50	23	0.36	51.2
5	208.0	142.0	68.3	1.02	0.70	13.55	23	0.48	68.1
6	206.2	138.8	68.2	1.02	0.63	15.56	27	0.36	57.1
7	200.0	125.0	62.5	1.10	0.56	17.20	14	0.38	68.3
8	187.5	112.5	60.0	1.06	0.43	22.58	15	0.32	74.5
				Normal plants					
0	31.0	22.5	72.6	1.27	0.41	0.77	20		
1	78.3	63.1	80.8	1.42	0.45	2.41	25		
2	139.5	118.9	85.2	(2.06)	0.43	5.63	27		
3	191.2	150.0	78.5	0.82	0.39	9.61	20		
4	250.0	180.0	72.0	0.61	0.30	13.70	15		
5	259.0	171.5	66.3	0.61	0.26	19.27	16		
6	280.0	178.0	63.5	0.49	0.15	20.42	12		
7	263.0	150	57.0	0.58	0.14	22.90	12		
8	250.0	133.1	53.4	0.58	0.17	30.40	9		

from 0.1 mg. for the 1st week preparation to a maximum of 2.86 mg. for the 5th week preparation and then also dropped off. The yield of virus protein thus paralleled the protein nitrogen content, and the fact that well over half of the protein nitrogen present in badly diseased plants was isolated by chemical methods as crystalline virus protein indicates the close relationship between the two. The nitrogen content of the dried pulp indicates that much nitrogen was in an insoluble form and remained in the pulp. The pulp from the diseased plants contained more insoluble nitrogen than did the pulp from normal plants. The decrease in the green weights of plants after 5 or 6 weeks was due to the drying up of some of the leaves near the bottoms of the plants. The yield of the first extract or juice based on the green weight of the plants averaged about 70 per cent.

In the second experiment, 52 flats each containing twenty-four Turkish tobacco plants about 2 inches high were used. All of the plants were inoculated on December 9, 1936, by rubbing two leaves of each plant twice with a bandage gauze pad which was kept well moistened with a solution of 0.1 M phosphate buffer at pH 7 containing 10^{-4} gm. of tobacco mosaic virus protein per cc. At the end of 1 week the plants in twelve of the flats were cut and frozen. As the remaining plants grew larger, a smaller number of flats of plants were cut at the end of weekly intervals up to and including 8 weeks, and also at the end of 13 weeks. These plants were placed in the freezing room. 5 weeks after beginning the experiment the plants from the first four harvestings were removed and put through a meat grinder. Disodium phosphate was added and the juice from each set of plants was pressed out and analyzed as previously described. The remaining five sets of plants were treated in a similar manner at weekly intervals. The nine portions of juice were filtered through Carl Schleicher and Schüll No. 1450½ paper in a room held at about 4°, and the virus protein present in one-half of each portion was purified and isolated by two successive centrifugations in the quantity ultracentrifuge, according to the method used for ring spot virus protein (9). The remaining halves of each portion of juice were purified by two successive precipitations with from 14 to 18 per cent ammonium sulfate and one precipitation in the quantity ultracentrifuge. The protein obtained from plants following

1, 2, and 13 weeks infection required less ammonium sulfate for precipitation than did protein obtained from plants infected for from 3 to 8 weeks.

The analytical data for the second experiment, in which the virus protein was isolated by means of the quantity ultracentrifuge as well as by chemical methods, are recorded in Table II. The gradual increase and eventual decrease in the green weight of the plants, the yield of about 70 per cent of the weight as juice, the tendency for the total nitrogen content of the juice to remain constant at about 1 mg. per cc., and the gradual increase and decrease of the protein nitrogen content are similar to the results obtained in the first experiment. The change in the amount of protein nitrogen remaining in the supernatant liquid following the first centrifugation of the juice (Table II, Column 7), with respect to the protein nitrogen content of the juice before centrifugation (Table II, Column 6), is especially noteworthy. Centrifugation serves to precipitate the virus protein and colloidal matter to the bottom of the tube in the form of a solid pellet, with the result that the inactive low molecular weight protein remains in the supernatant liquid. When there is but little high molec-

TABLE II

Preparation by Means of Quantity Ultracentrifuge of Virus Protein from Plants Harvested at Weekly Intervals Following Infection with Tobacco Mosaic Virus. Summary of Data

Time after inoculation (1)	Green weight per flat (2)	Juice per flat (3)	Yield of juice (4)	Total N per cc. juice (5)	Protein N per cc. juice (6)	Protein N in supernatant after first centrifugation (7)	Virus protein N per cc. juice (8)	Protein N recovered as virus protein (9)
wks.	*gm.*	*cc.*	*per cent*	*mg.*	*mg.*	*mg. per cc.*	*mg.*	*per cent*
1	130	94	72	0.81	0.39	0.33	0.06	15.8
2	238	181	76	0.89	0.56	0.27	0.18	31.8
3	363	263	73	1.08	0.70	0.25	0.34	49.3
4	425	319	75	1.08	0.74	0.25	0.37	49.5
5	476	358	75	1.08	0.84	0.22	0.46	54.5
6	460	352	76	1.3	1.00	0.20	0.42	41.4
7	580	420	73	1.14	0.78	0.16	0.37	47.9
8	525	390	74	1.19	0.83	0.11	0.37	45.0
13	500	300	60	1.12	0.77	0.27	0.15	19.3

ular weight virus protein present and most of the protein consists of inactive low molecular weight material, as was the case with the 1 week preparation, only a little protein is sedimented and most of the protein remains in the supernatant liquid. Thus, as may be seen from Table II, of the 0.39 mg. of protein nitrogen present per cc. in the 1st week juice only 0.06 mg. per cc. was sedimented on centrifugation and 0.33 mg. per cc. remained in the supernatant liquid. In this case the yield of virus protein nitrogen was only 15 per cent of the total protein nitrogen. However, practically all of the sedimented protein nitrogen was isolated as virus protein, hence this preparation contained practically no colloidal material. In the case of the juice from plants diseased for 5 weeks, only 0.22 mg. of protein nitrogen per cc. remained in the supernatant liquid on centrifugation and 0.62 mg. per cc. was sedimented to the bottom of the tube. The yield of virus protein nitrogen recovered from the total protein nitrogen was increased to 54.5 per cent, yet owing to colloidal material and a black substance which did not go back into solution the amount of virus protein nitrogen isolated (0.46 mg. per cc.) was only 74 per cent of the protein nitrogen which was sedimented. The amount of the black material, which becomes insoluble on sedimentation, was very noticeable in the case of the 13 weeks preparation. Only 30 per cent or 0.15 mg. per cc. of virus protein nitrogen was recovered from the 0.5 mg. per cc. of protein nitrogen which was sedimented. The amount of the black insoluble material increased as the amount of virus protein decreased. Although it may consist of plant pigment which increases as the plant grows older, it also seems possible that it may consist of a breakdown product of virus protein. It is preferable to work with plants cut only 3 or 4 weeks following inoculation in order to avoid the presence of this material.

In both experiments the amount of virus protein per cc. of juice was found to reach a maximum 5 weeks after inoculation. However, the rate of increase was greatest during the first 3 weeks following inoculation. The yields obtained from the portions purified by two precipitations with ammonium sulfate and one centrifugation are not given but were about the same as the yields obtained by centrifugation alone. The yields reported do not represent maximum yields, for in the earlier work with the quantity ultracentrifuge thin walled celluloid centrifuge tubes were used and much protein was lost through the bottoms of these tubes. Recently tubes having thicker walls have become available, and when such tubes are used the loss of protein is eliminated or considerably reduced. For example, with use of such tubes and juice obtained from badly diseased plants, it has been possible by means of two centrifugations to obtain about 80 per cent of the protein nitrogen in the juice in the form of crystalline virus protein. This demonstrates that only a small portion of the soluble protein nitrogen in such plants is not in the form of virus protein. This is an indication that the synthesis of low molecular weight normal protein is inhibited or that normal protein is destroyed or utilized for conversion into virus protein. If the conversion of normal plant protein into virus protein occurs, it is accompanied by a change in the antigenic properties, for normal protein and virus protein have not been found to give cross serological reactions (10, 11).

Virus Activity of Protein—The virus activity of the eight different preparations of protein in the first experiment was determined by means of the half leaf method and the results were analyzed in order to determine whether or not there were significant differences (12). It was found that there was an irregular significant variation in the activity. It is possible that this variation was due to differences arising during the chemical treatment and to the fact that the plants were worked up at different times. Therefore, in the second experiment the virus protein used for activity measurements was prepared by means of the quantity ultracentrifuge and an attempt was made to keep all treatments as nearly comparable as possible. The activity measurements, which are presented in Table III, were made by comparing the activity of all of the other preparations against that of the 4 weeks preparation by means of the half leaf method. It may be seen that there is no significant difference in the virus activity of protein obtained from plants following 2 to 13 weeks infection, but that the activity of protein obtained from plants only 1 week after infection is significantly less than that of the other preparations. The biological activity is one of the properties of the virus protein which is most sensitive to changes in the protein, and the fact that this property was not measurably changed in plants over a 12 week period is an indication of remarkable constancy of the virus protein in the host. An indication that slight changes do occur either in the plant or on isolation was obtained by Wyckoff (8) in a study of the sedimen-

TABLE III

Comparison of Virus Activity of Protein Prepared by Means of Quantity Ultracentrifuge from Turkish Tobacco Plants at Weekly Intervals Following Infection with Tobacco Mosaic Virus

No. of wks. of infection	Concentration of virus protein, gm. per cc.											
	10^{-4}						10^{-5}					
	Test 1			Test 2			Test 1			Test 2		
	Average No. of lesions per half leaf	No. of half leaves used	M.D.* / S.E.	Average No. of lesions per half leaf	No. of half leaves used	M.D.* / S.E.	Average No. of lesions per half leaf	No. of half leaves used	M.D.* / S.E.	Average No. of lesions per half leaf	No. of half leaves used	M.D.* / S.E.
1 4	86.2 97.5	58	2.2	53.0 86.2	57	8.7	42.2 53.2	61	3.3	30.6 39.3	56	3.1
2 4	96.0 101.5	59	1.2	84.4 83.6	56	0.0	58.0 67.0	45	3.8	43.3 48.5	55	1.2
3 4	94.0 91.2	56	0.5	107.0 98.4	45	1.5	48.4 50.0	56	0.5	57.6 46.2	41	3.1
5 4	96.0 109.5	58	2.8	48.5 45.8	60	1.0	44.3 45.8	57	0.7	54.5 52.0	55	0.7
6 4	77.0 73.0	54	0.9	43.0 44.0	58	0.3	30.5 32.3	60	0.3	54.5 46.8	52	3.0
7 4	59.5 59.5	57	0.0	103.0 95.0	52	1.2	54.3 62.6	55	1.9	26.2 23.4	60	1.5
8 4	38.8 45.2	60	2.0	90.3 75.0	60	3.3	45.3 39.4	59	2.2	43.4 39.0	60	1.8
13 4	109.0 113.0	60	1.1	122.0 105.5	60	2.7	52.6 49.5	54	1.1	46.3 49.2	60	0.5

* To show a significant difference between the mean number of lesions in any one experiment, the ratio of the mean difference (M.D.) to the standard error of the mean difference (S.E.) should not be less than 2.1.

tation constants of the protein preparations used for the activity measurements just described. However, the constancy of the activities and sedimentation constants of the various preparations demonstrates that the protein undergoes but little change in the plant over a long period of time and that it is possible by careful treatment to isolate essentially identical preparations of protein at different times. The fact that the virus activity of protein isolated from plants after 1 week's infection is significantly lower is quite interesting. Wyckoff (8) found this protein to be homogeneous with respect to molecular weight, hence it seems unlikely that the low activity is due to inactive partially built up molecules of virus protein.

Rate of Increase of Virus Protein in Inoculated Leaves—Although it is obvious from the results presented in Tables I and II that tobacco mosaic virus protein increases rapidly in inoculated plants, it is difficult to be certain of the magnitude of the increase due to the lack of data concerning the amount of protein actually introduced into the cells as inoculum. It is known that a portion remains on the inoculating pad and that another portion remains on the leaf surface. In order to secure data concerning the amount of virus protein actually serving as inoculum, all of the leaves of a group of Turkish tobacco plants about 2½ feet high and proved to be free of virus by subinoculation from the tip leaves were rubbed twice with a bandage gauze pad which was kept thoroughly moistened with a solution consisting of 100 cc. of 0.1 M phosphate buffer containing 10^{-4} gm. of tobacco mosaic virus protein per cc. The leaves were then well washed by sprinkling from above and the leaves from one-fifth of the plants were immediately removed and frozen. Similar sets of leaves were removed and frozen 9 hours, 1, 2, and 4 days after inoculation. The five sets of leaves were put through a meat grinder, disodium phosphate was added as previously described, and the five portions of juice were pressed out and filtered through thin layers of Standard celite. The virus activity of these preparations was determined and then 100 cc. portions were purified by means of four successive centrifugations in the quantity ultracentrifuge and taken up in 1 cc. portions of 0.1 M phosphate buffer at pH 7. 0.5 cc. portions were used for activity determinations and the remaining 0.5 cc. portions were treated with hot trichloroacetic acid. The preparations from leaves cut immediately, after 9, and after 24 hours gave no turbidity on addition of trichloroacetic acid, and hence contained less than 0.01 mg. of protein per cc. The 2 day preparation gave a slight turbidity, and hence contained about 0.01 mg. of protein per cc. The 4 day preparation was opalescent and on precipitation with trichloroacetic acid gave a good protein precipitate.

The amounts of protein obtained by trichloroacetic acid precipitation agree with the amount of protein which might be expected to be present as judged from the activity measurements which are presented in Table IV. The juice obtained from the leaves of plants removed immediately and 9 hours after inoculation contained practically no virus, and that obtained from the leaves of plants removed 24 hours following inoculation contained no demonstrable virus. However, when the latter juice was purified and concentrated 100 times, it was possible to demonstrate the presence of virus. The results indicate that more virus is present immediately after inoculation than after 9 or 24 hours, and that more virus is present after 9 hours than after 24 hours. After 2 and 4 days the amount of virus protein present was sufficiently large to be measurable by chemical tests. It seems probable that much of the virus obtained immediately and 9 hours following inoculation is due to virus on the leaf surfaces that was not removed when the leaves were washed. This virus protein would be expected to be in the form of a thin film which would be

TABLE IV

Virus Activity Measurements of Extracts from Inoculated Turkish Tobacco Leaves Removed at Different Intervals of Time Following Inoculation with a Solution Containing 10^{-4} Gm. of Tobacco Mosaic Virus Protein per Cc.

Time interval following inoculation	0	9 hrs.	1 day	2 days	4 days
Extract tested directly	0.1*	0.2	0.0	8.0	46.5
" " after 4 supercentrifugations and 100 times concentration	55.0	10.1	1.8	22.0	128.5

* The numbers represent the average number of lesions per leaf obtained on ten or more leaves of *Phaseolus vulgaris*, var. Early Golden Cluster on inoculation with the designated preparation.

subject to rather rapid denaturation, hence most of the protein on the surface of the leaves should be inactivated after 24 hours owing to denaturation. This would serve to explain the initial decrease in the amount of demonstrable virus. It seems probable that the amount of virus obtained after 24 hours is more nearly a measure of the amount of virus actually introduced into the cells than is the amount obtained immediately or after 9 hours. Therefore, in this experiment it is preferable to use the amount of virus demonstrable after 1 day as a measure of the amount originally introduced into the cells. Although this amount is undoubtedly much greater than that actually introduced, the amount is still so small that it is impossible to measure it chemically, but, on the basis of the activity measurements, it corresponds to about 10^{-11} gm. of protein per cc. of juice. Therefore, of the 2 mg. of protein which were applied to the leaves, only about 10^{-6} mg. or about one-millionth was recovered. If one considers the multiplication during the 1st day and the fact that after 4 days approximately 1 mg. of virus protein was recovered, it may be estimated that over a 4 day period the virus protein in inoculated leaves was increased over a million times.

The considerations just described are based on the assumption that the virus isolated represented the amount actually in the plant material. Although it is known that when fairly large amounts of virus protein are present about 90 per cent of the virus protein can be removed by one extraction, there are no data concerning the efficiency of extraction when very small amounts of protein are present. It seemed desirable, therefore, to add known small amounts of virus protein to the pulp and to the juice from normal plants and to determine the amount of protein which could be recovered. Accordingly, 2 mg. portions of virus protein were added to 100 gm. of pulp and to 100 cc. of juice from normal plants. The juice was pressed from the pulp and filtered through a thin layer of Standard celite. The juice obtained, the 100 cc. portion of juice containing 2 mg. of virus protein, and a 100 cc. portion of the juice from normal plants were centrifuged three successive times in the quantity centrifuge in a field of about 60,000 times gravity. No virus protein was obtained from the normal juice preparation to which no virus protein had been added, 40 per cent of the virus protein was recovered from that added to the pulp, and about 75 per cent was recovered from that added to normal juice. This experiment demonstrates that, even when there is only 0.00002 gm. of virus protein present per gm. of pulp or of juice, 40 and 75 per cent, respectively, of the virus protein can be recovered. Although this indicates that much of the virus protein may be recovered when amounts of about 10^{-5} gm. or greater per gm. of pulp or of juice are concerned, it is problematical as to the amount that may be recovered when still smaller amounts of virus protein are present. Although it is impossible to measure smaller amounts of virus protein chemically, it is quite possible to estimate such amounts biologically.

In order to obtain some idea of the amount of virus protein recoverable when such small amounts are concerned, 1 part of virus protein was added to 10 million parts of pulp. The juice was pressed out, diluted 1:10 with 0.1 M phosphate buffer at pH 7, and tested for virus activity by the half leaf method against similar dilutions of juice from normal plants to which known amounts of virus protein had been added. The diluted extract of the pulp produced about the same number of lesions as did the diluted juice preparation containing 5×10^{-9} gm. of virus protein per cc., hence the activity of the extract of the pulp was about equal to that of juice containing about one-half the amount of virus protein originally added to the pulp. As a whole, these experiments indicate that there is justification for considering that most of the virus protein present in plants may be obtained in the extract of such plants, even though the amount of virus protein may represent less than a millionth part of the plants.

The writer is greatly indebted to Dr. Ralph W. G. Wyckoff for the use of an air-driven quantity centrifuge during the course of the work.

SUMMARY

The increase of tobacco mosaic virus protein in Turkish tobacco plants has been determined by isolating the virus protein in plants diseased for different periods of time. The efficiency of the isolation technique was determined by isolating virus protein from artificially prepared mixtures containing known amounts of virus

protein. It was found that about 40 per cent of the virus protein can be isolated from plants containing only about 1 part of virus protein per 100,000 parts of plant material. Virus protein in Turkish tobacco plants was found to increase from an estimated 10^{-6} mg. per gm. of plant material to about 3 mg. per gm. of plant material during the course of 5 weeks. Virus protein in inoculated leaves was estimated to increase over a million times during a 4 day period. Although the virus protein content was found to reach a maximum 5 weeks after inoculation, the rate of increase was found to be greatest during the first 3 weeks. The total nitrogen content of extracts of diseased plants was found to remain about constant over long periods of time, whereas the protein nitrogen content was found to increase and reach a maximum and then to decrease. The amount of low molecular weight protein was found to decrease as the amount of the virus protein increased. No significant difference was found in the virus activity of protein obtained from plants that had been infected for from 2 to 13 weeks, although that of protein obtained from plants infected for only 1 week was significantly less.

BIBLIOGRAPHY

1. Holmes, F. O., *Am. J. Bot.*, **17**, 789 (1930); *Contrib. Boyce Thompson Inst.*, **4**, 297 (1932).
2. Böning, K., *Z. Parasitenk.*, **1**, 198 (1928).
3. Samuel, G., *Ann. Appl. Biol.*, **21**, 90 (1934).
4. Caldwell, J., *Ann. Appl. Biol.*, **18**, 279 (1930); **21**, 191 (1934).
5. Stanley, W. M., *J. Biol. Chem.*, **115**, 673 (1936).
6. Loring, H. S., and Stanley, W. M., *J. Biol. Chem.*, **117**, 733 (1937).
7. Wyckoff, R. W. G., Biscoe, J., and Stanley, W. M., *J. Biol. Chem.*, **117**, 57 (1937).
8. Wyckoff, R. W. G., *J. Biol. Chem.*, **121**, 219 (1937).
9. Stanley, W. M., and Wyckoff, R. W. G., *Science*, **85**, 181 (1937).
10. Stanley, W. M., *Phytopathology*, **26**, 305 (1936).
11. Seastone, C. V., Loring, H. S., and Chester, K. S., *J. Immunol.*, in press.
12. Loring, H. S., unpublished work.

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THE ULTRACENTRIFUGAL ISOLATION OF LATENT MOSAIC VIRUS PROTEIN

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Plate 2

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The concentration and purification of tobacco mosaic virus protein (1, 2) with the quantity ultracentrifuge has led to the isolation of other plant virus proteins by this method. A high molecular weight protein has thus been obtained from the clarified juice of plants infected with tobacco ring spot virus, and preliminary experiments with juices of plants having severe etch and latent mosaic of potato have shown that similar heavy proteins are associated with these diseases (3). Further experiments have now been performed on the application of these ultracentrifugal procedures to the concentration, purification, and characterization of the high molecular weight protein which is present in plants infected with the latent mosaic virus. This paper presents the results of these experiments and provides data showing the approximate yields of the protein from diseased *Nicotiana tabacum*, L., var. Turkish and *Nicotiana glutinosa*, L., plants.

Several strains of latent mosaic virus are recognized. That used in the present experiments was the potato ring spot strain described by Johnson (4). It had been carried in *Nicotiana glutinosa* and *Nicotiana tabacum* for several years and gave typical symptoms of the potato ring spot disease. Plants of *Nicotiana glutinosa* and Turkish tobacco were grown in a greenhouse either in pots or in shallow flats and were inoculated when from 4 to 6 inches high with diluted juice from a latent mosaic-diseased plant. They developed typical symptoms and were harvested from 2 to 6 weeks after inoculation. When cut they were placed overnight in a refrigerator at −14° and the next day ground in a meat chopper. The frozen pulp was mixed with 4 per cent by weight of K_2HPO_4, allowed to thaw at room temperature, and the cold juice strained through four layers of bandage gauze.

This strained juice was finally clarified by gravity filtration through No. 1450½ Carl Schleicher and Schüll filter paper in a room held at about 4°.

The clear filtrate was ultracentrifuged for 1½ hours in quantity heads of 100 to 150 cc. capacity in a field of about 60,000 times gravity (5, 6). The solution and the centrifuge head, which were cooled to 0° before the centrifuge was started, warmed up less than 10° during a run. The upper 50 per cent of the supernatant liquid was pipetted off, analyzed for protein, and tested for virus activity. The remainder was poured off; the pellets that had formed were immediately covered with 0.1 M phosphate buffer at pH 7, and the tubes containing them were placed on ice. The pellets were dissolved in about one-tenth the original volume and the solution was centrifuged in an angle centrifuge to remove aggregated colloidal matter. The supernatant liquid containing the soluble protein was again ultracentrifuged; the supernatant liquid was removed and analyzed as before, and the pellet put back in solution. The cycle of low speed centrifugation to remove aggregated matter and ultracentrifugation to sediment the virus was repeated a third time. The pellets from this third ultracentrifugation dissolved in 0.1 M phosphate buffer gave whitish opalescent solutions which, as shown by the analytical ultracentrifuge, contained very little except high molecular weight protein. A 0.1 per cent solution gave the usual color reaction for proteins with Millon's test, the xanthoproteic reaction, and Folin's phenol reagent.

Analytical data showing the protein concentration of the juice before and after ultracentrifugation and the yields of the heavy protein obtained from several different batches of *Nicotiana tabacum* and *Nicotiana glutinosa* plants are summarized in Table I. The yields varied considerably, but their averages (4.9 and 10.5 mg. per 100 cc. of juice, respectively) indicate a higher concentration of virus protein in *Nicotiana glutinosa* than in *Nicotiana tabacum* plants. The examination in the analytical ultracentrifuge (6, 7) of the solution of the pellets from the first, second, and third ultracentrifugations gave results in accord with the chemical analyses of the supernatant liquids from each. Absorption photographs of the solution of the pellet from the first ultracentrifugation have shown (Fig. 1) the sharp sedimenting boundary of the heavy protein; sometimes, however, it was still obscured by the normal low molecular weight proteins and the pigment that remained. The sedimentation diagram from a solution of the pellet from the second ultracentrifugation (Fig. 2) exhibited a considerable amount of "unsedimentable" ultraviolet-absorbing material still contaminating the heavy protein; if the plant juice was carefully prepared and the extraction properly carried out, the diagram (Fig. 3) from a solution of the pellet from the third ultracentrifugation was that of the pure heavy protein.

The relative activities of the purified high molecular weight protein, of the supernatant liquid after ultracentrifugation, and of the original juice were compared by counting the number of primary lesions produced on whole leaves of young Turkish tobacco plants over the range of dilutions at which infection was obtained. Dilutions were prepared in 0.1 M phosphate buffer at pH 7. The results with juice and with preparations of virus protein from both *Nicotiana tabacum* and *Nicotiana glutinosa* are shown in Table II. The method of inoculation was the same as that used for tobacco mosaic virus protein. The upper half of the supernatant liquid after ultracentrifugation usually contained sufficient virus to give infection and in some instances was also infectious in a dilution of 1:10. Since the original juice quite regularly gave infection at a dilution of 1:10,000, ultracentrifugation removed about 99.9 per cent of the virus activity from the upper half of the supernatant liquid. The purified

TABLE I

Ultracentrifugation of Juice from Nicotiana tabacum and Nicotiana glutinosa Extracted with K_2HPO_4. Protein Content of Juice, Successive Supernatant Liquids, and Yields

Host plant	Volume of juice centrifuged	Protein concentration, mg. N × 6 per cc.*			Total yield	Percentage yield, mg. per 100 cc. juice
		Juice	Supernatant of 1st centrifugation	Supernatant of 2nd centrifugation		
	cc.				*mg.*	
N. tabacum	176	2.88	2.49	0.42	15	8.5
" "	800	3.23	2.7	0.51	14	1.8
" "	176	1.92	1.79	0.30	11	6.3
" "	400	2.14	2.08		11	2.8
" *glutinosa*	300	3.5	2 68		33	11.0
" "	100	2.74	2.13	0.29	10	10.0

* The supernatant liquid of each third centrifugation gave a faint cloudiness with $CHCl_3COOH$.

protein proved to be infectious at a dilution of 1:10^8, equivalent to 10^{-8} gm. of protein per cc.; the virus protein isolated is, therefore, between 1000 and 10,000 times more infectious than the original juice. A comparison of the relative numbers of lesions obtained with undiluted juice and a virus protein concentration of 10^{-4} gm. per cc. indicates, in agreement with the amount of virus protein actually isolated, that the concentration in the juice is of the order of 10^{-4} gm. per cc. From these results it is apparent that the concentration of latent mosaic virus protein in the juice of diseased plants lies between that for tobacco mosaic (8) and that for tobacco ring spot (3) virus proteins, which have been shown to be approximately 3 mg. per cc. and 0.01 mg. per cc., respectively.

TABLE II

Relative Activities of Purified Latent Mosaic Virus Protein and Juice from Nicotiana tabacum and Nicotiana glutinosa

	No. of leaves	Concentration, gm. protein per cc.				
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Virus protein from *N. tabacum*	24	326*	46.6	15.8	0.3	0
" " " " *glutinosa*	12	117	74.8	11.8	2.9	1.3
	12	88	24.8	5.3	0.75	0.33
		Dilution of juice				
		Undiluted	1:10	1:100	1:1000	1:10,000
Unfiltered juice from *N. tabacum*	12	335	127	31.4	4.6	0.3
" " " " *glutinosa*	12	357	144	57.6	6.8	1.3

* Average numbers of lesions on whole leaves of young Turkish tobacco plants.

The ultracentrifugal analyses of solutions of the purified latent mosaic virus protein show the series of sharply sedimenting boundaries characteristic of molecules homogeneous with respect to size and shape (Fig. 3). These molecules sediment much more slowly than those of the tobacco mosaic virus protein and hence are smaller. In order to establish their sedimentation constant, several series of photographs, like those given in Fig. 3, have been made from proteins prepared from different groups of plants. In each series the individual photographs were made at intervals of exactly 5 minutes, the field at the center of the cell ranging between 17,000 and 30,000 times gravity. The optimum protein concentration is practically the same as for the tobacco mosaic virus protein: for a 2 mm. thick cell it is 1.8 mg. per cc. The customary analysis of the sedimentation diagram gives values for the sedimentation constants that are closely grouped about a mean of $s_{20°} = 113 \times 10^{-13}$ cm. sec.$^{-1}$ dynes^{-1}. This is to be contrasted with the larger $s_{20°} = 174 \times 10^{-13}$ characteristic of the tobacco mosaic virus protein (2, 9). The molecular weight of the latent mosaic virus protein, of course, cannot be computed from this sedimentation constant unless the diffusion constant is known. This has not yet been determined, but if the general progression of diffusion constants with sedimentation constants observed in Svedberg's results (10) applies to the latent mosaic protein, then it might be expected to be of the order of 1.3×10^{-7}, the exact value being strongly influenced by the molecular shape. A molecule with $s_{20°} = 113 \times 10^{-13}$ and $D = 1.3 \times 10^{-7}$ would have a molecular weight of about 9 millions.

The latent mosaic virus protein prepared by the method outlined above commonly shows a second faint but sharp boundary which settles more rapidly than the principal one. In this respect too it resembles the tobacco mosaic virus protein. It can be shown that for the tobacco mosaic virus protein this second and "heavier" molecular component is a derivative of the principal one; and there is reason to believe that the same thing is true with the latent mosaic protein. The sedimentation constant of this second boundary is $s_{20°} = 131 \times 10^{-13}$.

SUMMARY

The application of the quantity and analytical ultracentrifuges to the purification and characterization of the virus protein which is associated with the latent mosaic disease of potato in *Nicotiana glutinosa* and *Nicotiana tabacum* plants is described. The results of chemical analyses of the supernatant liquids following ultracentrifugation and ultracentrifugal analyses of purified preparations show that a homogeneous high molecular weight protein is obtained after three ultracentrifugations. Preparations made by the method outlined show a principal boundary with $s_{20°} = 113 \times 10^{-13}$ cm. sec.$^{-1}$ dynes^{-1} and usually a second faint boundary with $s_{20°} = 131 \times 10^{-13}$. The protein is present to the extent of about 0.02 to 0.1 mg. per cc. of the juice of infected plants and was found to reach a somewhat greater concentration in diseased *Nicotiana glutinosa* than in *Nicotiana tabacum* plants.

The latent mosaic virus protein was found to be between 1000 and 10,000 times more infectious than the original juice.

BIBLIOGRAPHY

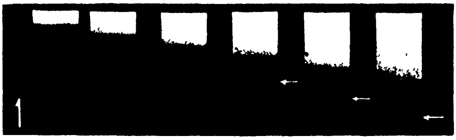
1. Wyckoff, R. W. G., and Corey, R. B., *Science*, **84**, 513 (1936).
2. Wyckoff, R. W. G., Biscoe, J., and Stanley, W. M., *J. Biol. Chem.*, **117**, 57 (1937).
3. Stanley, W. M., and Wyckoff, R. W. G., *Science*, **85**, 181 (1937).
4. Johnson, J., *Wisconsin Agric. Exp. Stat. Research Bull., No. 63* (1925).
5. Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, **64**, 303 (1936).
6. Wyckoff, R. W. G., and Lagsdin, J. B., *Rev. Scient. Instruments*, **8**, 74 (1937).
7. Svedberg, T., *Naturwissenschaften*, **22**, 225 (1934). Biscoe, J., Pickels, E. G., and Wyckoff, R. W. G., *J. Exp. Med.*, **64**, 39 (1936).
8. Stanley, W. M., *J. Biol. Chem.*, **121**, 205 (1937).
9. Eriksson-Quensel, I., and Svedberg, T., *J. Am. Chem. Soc.*, **58**, 1863 (1936). Wyckoff, R. W. G., *J. Biol. Chem.*, **121**, 219 (1937).
10. Svedberg, T., *Chem. Rev.*, **20**, 81 (1937).

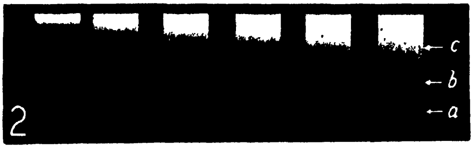
EXPLANATION OF PLATE 2

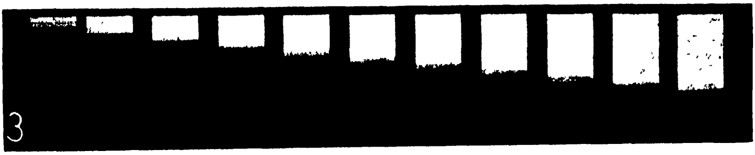
FIG. 1. The sedimentation diagram of a solution of the impure virus protein obtained from the pellet of the first ultracentrifugation. The contaminating light proteins cause the liquid immediately below the air bubble at the very top of the pictures to be less transparent than the bubble itself. The barely visible sharp boundary indicated by arrows in the last pictures corresponds to a sedimentation constant (uncorrected for the viscosity of the contaminating "unsedimentable" proteins) of $s_{20^\circ} = 117 \times 10^{-13}$ cm. sec.$^{-1}$ dynes^{-1}; the more conspicuous diffuse one above it gives $s_{20^\circ} = 79 \times 10^{-13}$ cm. sec.$^{-1}$ dynes^{-1}.

FIG. 2. The sedimentation diagram of the partially purified virus protein obtained by dissolving the pellet from the second ultracentrifugation. The lack of contrast between the air bubble and the top of liquid indicates that very little "unsedimentable" protein remains. The sharp and most rapidly sedimenting boundary (*a*) corresponds to $s_{20^\circ} = 115 \times 10^{-13}$; the light diffuse boundary (*c*), which is not due to the virus, gives $s_{20^\circ} = 37 \times 10^{-13}$. Between these two there is an exceedingly faint boundary (*b*) which measures $s_{20^\circ} = 71 \times 10^{-13}$. The nature of the substances causing these lighter boundaries is not clear, for they are not always present in concentrates from plants diseased with latent mosaic and they are not carriers of the virus activity.

FIG. 3. The sedimentation diagram of a solution of the latent mosaic virus protein purified by three ultracentrifugations. All the lighter impurities have been eliminated. The only two boundaries present are the principal one with $s = 115$ and the faint secondary one with $s = 130$. The pattern remains unchanged if the protein is subjected to additional ultracentrifugations.







(Loring and Wyckoff: Latent mosaic virus protein)

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